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THE EFFECTS OF CARBOHYDRATE AND AMINO ACIDS ON MUSCLE PROTEIN SYNTHESIS AFTER ACUTE RESISTANCE EXERCISE AND MUSCLE ADAPTATION FOLLOWING CHRONIC RESISTANCE TRAINING

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Resistance exercise (RE) is purported to induce muscle protein accretion primarily by stimulating muscle protein synthesis (MPS), with its effect potentiated by providing a protein or amino acid (AA) supplement post exercise. Glutamine, a conditionally essential AA, is increasingly recommended to improve exercise performance, but it is poorly soluble and unstable in sports drinks. This limitation can be overcome by combining L-glutamine with L-alanine to form a dipeptide (AlaGln). The first study demonstrated that AlaGln supplementation post resistance exercise significantly reduced the phosphorylation of AMPK and NF-kB p65 without activating intracellular signals for MPS. This study also showed that whey protein (WP) activated the mTOR signaling pathways without affecting signaling proteins that controls muscle protein breakdown (MPB). The results provide indirect evidence that AlaGln blocks

MPB via suppressing the activation of AMPK-FOXO3A and NF-kB p65, while WP promotes MPS.

Recent research suggests that adding carbohydrate (CHO) to a protein supplement post exercise can produce greater protein accretion and subsequently increase the magnitude of resistance training adaptation relative to protein supplementation alone. Early studies from our laboratory found that co-ingestion of CHO and protein, as compared with CHO or protein intake individually, had a greater effect on the activation of anabolic signaling proteins. However, the effect of CHO plus protein on MPS and muscle adaptation is controversial. The second study demonstrated that adding CHO to a protein supplement (CP) accelerated MPS via activating the mTOR-signaling pathway in comparison with placebo (PLA) and WP during early exercise recovery, but CP did not affect signaling proteins that regulate MPB. The third study found that CP enhanced muscle strength relative to PLA and WP. The greater strength development in CP appeared due to increased myofibrillar protein content. Increased muscle hypertrophy occurred without an increase in myonuclei suggesting satellite cell activation was not required for muscle fiber development. Taken together, the results of this series of studies suggest that 1) AlaGln inhibits MPB following acute RE; 2) Adding CHO to a protein supplement accelerates muscle recovery by stimulating MPS, and increases the magnitude of muscle strength by accumulating more myofibrillar protein in comparison with PLA and WP.

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Chapter I: General Introduction

OVERVIEW

Skeletal muscle constitutes about 40% of body weight and contains 50-75% of all proteins in the body (10). An enhancement of muscle mass is positively correlated with increased muscle strength and power, so an increase in muscle mass is crucial for promoting mobility, quality of life, and prevention of life-style related diseases.

Two major strategies are generally used to increase muscle mass: resistance exercise (RE) and nutritional supplementation. RE is a potent stimulator of muscle protein synthesis (MPS), and its repeated activity can bring about skeletal muscle hypertrophy (14, 23). Muscle protein breakdown (MPB), however, is also elevated following RE. It results in a negative muscle net protein balance during the early recovery phase following RE if no nutritional supplement is consumed. This then may delay muscle repair and development, and extend the period of muscle fatigue.

It is well known that protein intake is essential for hastening muscle recovery by ensuring sufficient amino acid (AA) availability and activation of the mammalian target of rapamycin complex 1 (mTORC1, referred as mTOR below) signaling pathway. mTOR is a central kinase that integrates upstream signals from muscle contraction, AA, and growth factors to stimulate MPS via mediating mRNA translation initiation. Therefore, a single bout of RE is able to activate the mTOR signaling pathway, with its effects potentiated by providing a protein supplement following RE. However, not all AA equally contribute to the muscle protein accretion.

Glutamine is the most abundant AA in plasma and in skeletal muscle (15). It was found that the intramuscular glutamine concentration was highly associated with the rate of MPS (30). Therefore, maintaining or increasing the glutamine concentration in skeletal muscle is important, in particular under a catabolic state. Glutamine is classified as a conditionally essential AA. Under certain conditions such as severe illness, trauma,

cancer, and overtraining, the demand for glutamine exceeds its *de novo* synthesis and thus exogenous glutamine intake is necessary. In the past two decades, glutamine supplementation has become increasingly popular with athletes due to its potential benefit on glycogen resynthesis (5), protein synthesis (26, 30), and prevention of muscle atrophy (43). It also has been proposed that glutamine can exert a direct stimulatory effect on MPS possibly via the activation on the mTOR signaling pathway (29, 30), and inhibiting muscle atrophy via reducing the expression of myostatin, Atrogin-1 and MuRF-1 (29, 43). However, due to its poor solubility and instability in solution, interest from industry is greatly leaning to glutamine-containing dipeptides. Sustamine^{TM} (SUS), one such dipeptide composed of alanine and glutamine, has been shown to have better solubility and stability in a solution compared with glutamine alone (19, 41). L-alanylglutamine (AlaGln) administration has been suggested to attenuate muscle damage as indicated by lower inflammatory biomarkers following a prolonged endurance exercise (7). However, its role on signaling proteins that control MPS and MPB after an acute RE needs to be investigated.

Recent research also suggests that the addition of carbohydrate (CHO) to a protein supplement after RE may better benefit muscle recovery than that of protein/AA intake alone (9, 13, 35). We propose this idea because a carbohydrate/protein supplement greatly increases insulin secretion, and insulin is a very strong anabolic hormone. Immediately after high intensity RE, the human body is under a catabolic state due to the elevation of catabolic hormones such as cortisol and epinephrine. Insulin can inhibit the secretion of these hormones and reverse the catabolic environment to an anabolic physiological state. Also, many studies have clearly shown a potential suppressive effect of insulin on MPB (2, 6, 42) due to its inhibition on forkhead box 3A (FOXO3A) (12). Insulin also activates the mTOR signaling pathway through activation of Akt, but its role on MPS remains controversial. Many studies have utilized an isocaloric CHO supplement as a control in comparison with a protein supplement to study MPS post exercise. The effectiveness of the CHO supplement is generally negative or only mildly successful.

This failure to stimulate MPS is most likely due to a lack of AA availability. However, co-ingestion of CHO with protein is expected to maximize muscle protein accretion by overcoming a deficient AA pool and promoting a hyperinsulinemic state. To date, the few studies that have investigated the combined effects of CHO and protein supplementation on MPS have provided inconsistent results (28, 32). Moreover, results from some studies have demonstrated a similar pattern of change between MPS after acute RE and muscle hypertrophic response during a prolonged resistance training program (20, 22, 45), while other research has failed to support this relationship (31, 33). Thus, conducting a long-term study is necessary to examine the role of CHO in conjunction with protein on muscle hypertrophy and strength.

To address the incongruent information in the literature regarding nutritional supplementation and muscle protein synthesis and development, we aimed to investigate 1) the effect of AlaGln on cell signaling proteins that control MPS and MPB in comparison with exercise plus protein or exercise alone; 2) the effect of co-ingestion of CHO with protein compared with protein supplementation alone on MPS during the immediate hours after acute RE; 3) and the effect of co-ingestion of CHO with protein on muscle hypertrophy during a resistance training program.

OBJECTIVES

Study 1: to compare the effect of acute resistance exercise (RE) and post exercise supplementation of whey (WP) and two different dosages of AlaGln on rat skeletal muscle cell signaling proteins that control protein synthesis and degradation.

Study 2: to determine whether the addition of CHO to a WP supplement (CP) can increase MPS after acute RE compared with exercise plus WP or exercise alone (PLA=ddH_2O). We also proposed to investigate the relationship of MPS with phosphorylation of proteins responsible for control of MPS in the insulin and mTOR signaling pathways. Additionally, we investigated the effect of these treatments on the FOXO3A pathway that controls MPB.

Study 3: to determine whether a post-exercise CHO plus whey hydrolysate (CP) supplement compared to WP or PLA can bring about a greater enhancement of muscle hypertrophy and percent of lean body mass during 8 weeks of resistance training. We also aimed to investigate whether activation of satellite cells (SCs) was involved in the change of muscle mass by different treatments.

HYPOTHESES

Study 1

1. An acute RE will modulate phosphorylation of signaling proteins that increase MPS and reduce MPB compared with the non-exercise group (SED) at both 20 and 40 min post exercise.
2. AlaGln provided immediately post RE will modulate phosphorylation of signaling proteins in a manner that enhances muscle protein synthesis and reduces protein degradation compared with exercise alone (PLA) at both 20 and 40 min post exercise.
3. AlaGln provided immediately post RE will have a better or comparable effect on phosphorylation of signaling proteins in a manner that enhances muscle protein synthesis and reduces protein degradation in comparison with WP at either 20 or 40 min post exercise.
4. The high dose AlaGln will have a better effectiveness than the low dose AlaGln in the regulation of signaling proteins that increase MPS and reduce MPB at both 20 and 40 min post exercise.
5. AlaGln will increase anabolic hormonal circulation (insulin, GH, and IGF-1).

Study 2

1. In comparison with a WP supplement or PLA, a CP supplement will (a) enhance MPS after acute RE, and (b) increase the phosphorylation of cell signaling proteins in the mTOR and insulin signaling pathways.

2. In comparison with a WP supplement or PLA, a CP supplement will enhance modulation on the phosphorylation status of cell signaling proteins in the FOXO3A pathway.
3. In comparison with a WP supplement or PLA, a CP supplement will promote a higher anabolic hormonal circulation (insulin, GH, and IGF-1) and lower the corticosterone level in plasma of rats.

Study 3:

1. In comparison with PLA and WP, CP will demonstrate a greater improvement in the body composition (increasing lean body mass and/or decreasing fat mass) during 8 weeks of resistance training.
2. In comparison with PLA and WP, CP will produce greater muscle hypertrophy and muscle strength during 8 weeks of resistance training.
3. Additional nuclei as assessed by protein expression of Pax 7, MyoD, and myogenin will be recruited over 8 weeks of resistance training, and enhanced by CP supplementation.
4. The order of effects will be CP > WP > PLA.

SIGNIFICANCE

Acute RE induced MPS can last for up to 48 h (38), but MPB is simultaneously elevated after RE. Previous studies have demonstrated that exercise-stimulated MPS may not occur until 6 h post RE in rodents (21) or 3 h post RE in humans (38), whereas MPB can be increased during RE and remain elevated for 24 h post exercise in the fasted state (1, 38). Acceleration of muscle recovery after high-intensity RE is essential for athletes to maintain optimal performance in an upcoming competition, especially when the recovery time is limited between the two activities. Muscle recovery is also important after each high-intensity RE session during a prolonged exercise training regimen in order to generate greater muscle hypertrophy. On the other hand, protein intake alone increases

MPS with a minor effect on MPB, and its effect on MPS is transient. When combining protein supplementation with RE, their additive effects on the activation of the mTOR signaling pathway can augment the amplitude and duration of MPS. It is well established that increased AA availability post exercise is positively associated with an increase in MPS. However, not all AA contained in a protein supplement participate in the activation of mTOR, which in turn stimulates MPS. Glutamine is a conditionally essential AA. In normal situations, its requirements can be met by *de novo* synthesis within most tissues (e.g. skeletal muscle and liver) (36, 37) and from dietary protein intake. A fall in glutamine level can be seen in severe diseases, trauma, and strenuous exercise, in which exogenous glutamine intake may be necessary to meet the body's demands. Glutamine is the most abundant AA in plasma and occupies more than 60% of the intramuscular AA pool (11, 15). Therefore, maintenance of glutamine level is crucial for various metabolic reactions. Early research observed a positive correlation between intramuscular glutamine concentration and the rate of MPS (30, 39, 40). Several investigations indicated that exogenous glutamine stimulates MPS by means of increasing GH and activating the mTOR signaling pathway (18, 30), particularly under a catabolic state (29, 46). In addition to its possible benefit on MPS, glutamine may inhibit MPB (16, 43). Despite its important influence on muscle protein accretion, glutamine is not readily dissolvable in a sports drink due to its instability and limited solubility in solution with a low pH. Nevertheless, this limitation can be overcome by combining glutamine with another amino acid such as alanine (Sustamine™, a dipeptide composed of AlaGln). Consumption of AlaGln facilitates glutamine absorption and increases plasma glutamine concentration to a higher level (19, 41). AlaGln was reported to decrease muscle damage post exercise training and attenuate the reduction of MPS in post-operative patients (7, 17). However, it is not conclusive as to whether AlaGln has any impact on muscle protein metabolism after acute resistance exercise. Study 1 represented the first study to investigate the effect of AlaGln on cell signaling proteins controlling MPS and MPB. Demonstrating significant changes in the phosphorylation state of signaling proteins conducive with an increase in MPS and decrease in MPB with AlaGln supplementation

would support the use of AlaGln in sports recovery drinks and thus provide a practical means of improving muscle net protein balance after exercise.

With regard to protein intake, 20 to 25 g high-quality protein supplementation is sufficient to maximize MPS post RE in young adults (34). However, it has little or no effect on MPB. Recent research suggests that adding CHO to a protein supplement may have potential benefits on muscle recovery after RE (24, 32, 35). It is widely accepted that CHO intake can inhibit MPB by an increase in the phosphorylation of FOXO3A. In addition, insulin activates mTOR (35) through a different pathway from exercise or AA, suggesting that the combination of CHO and protein supplementation may have an additive effect on muscle protein accretion. Some *in vitro* studies demonstrated that insulin increases myotube protein synthesis (25, 27), but only a few *in vivo* studies have confirmed this positive effect (13). The few studies that have investigated the combined effects of CHO and protein on MPS also provided inconsistent results (28, 32). Therefore, in studies 2 and 3 we investigated whether CHO plus protein results in a greater MPS and training adaptation than protein alone. In our studies, dextrose (L-glucose) was chosen as the CHO source because dextrose is a simple CHO and promotes a high insulin response (3). With regard to the source of protein supplement, ingestion milk protein is superior to soy protein in stimulating protein synthesis after RE (20). Compared with casein, however, whey is considered the best option to provide immediately post exercise for rapid muscle recovery because it has a faster absorption rate than casein (4, 8). In studies 2 and 3, if dextrose combined with whey is able to enhance MPS and promote muscle hypertrophy, then this nutrient combination should be an effective supplement for athletes to hasten muscle recovery in order to perform optimally during competition and achieve a faster training adaptation.

Collectively, one major goal of our studies was to develop more effective ways (i.e. AlaGln and CP) to regulate cell signaling pathways controlling muscle protein accretion following acute strenuous RE. The other major goal of our study was to investigate the feasibility of a CP supplement on muscle protein synthesis and muscle

adaptation to RE. If our hypotheses are correct, our studies will provide several non-pharmacological strategies to accelerate muscle protein accretion and training adaptation. Ideally, these nutritional strategies will yield an enhancement of lean body mass and increase in strength, thereby improving physical function for athlete. The strategies will also improve the muscle development of young adults and reduce the rate of sarcopenia in middle-aged and older individuals and improve their quality of life.

LIMITATIONS AND DELIMITATIONS

Ladder climbing was used as a resistance exercise protocol in all three studies. Therefore, the first consideration was that rats refusing to climb would affect the experiment results. To minimize this limitation, we familiarized the rats with this task over the first two weeks. If the rats were unwilling to climb, we provided encouragement by tapping their tails lightly with a bottle brush. If they continued to refuse to climb, they were eliminated from the study. Second, the muscle development in the rat with ladder climbing is mainly in the small muscles that control the toes, which does not afford large changes in body composition.

Studies 1 and 2 were to test MPS and signaling proteins that control MPS and MPB. As we know, the phosphorylation of proteins is varied and occurs transiently as a result of muscle contraction or nutritional supplementation. Therefore, a third limitation is the possibility of missing the phosphorylation of designated proteins. According to the study of Harris et. al., ingestion of AlaGln led to a faster and higher increase in the plasma glutamine concentration than that of either glutamine or wheat protein 15-60 min after administration. The peak concentration of plasma glutamine caused by AlaGln supplementation is 30 min (19). Therefore, to minimize time course limitations, we chose two different time points (20 and 40 min) to investigate in study 1 post exercise. Based on the study of Morrison et al (35) and our pilot studies, we also chose two different time points (1 and 2 h) to investigate in study 2. However, each protein likely has a different pattern of phosphorylation, and therefore it is possible that our results are limited by the times of muscle harvesting.

To better understand the roles of AlaGln and CP on muscle protein accretion, both MPS and MPB should have been examined in our acute studies. However, due to funding and specific equipment limitations, we were only able to measure the signaling proteins that control MPS and MPB in study 1. Additionally, we could have measured 3-methylhistidine levels in urine, plasma and isolated muscle as a marker of MPB, but this method has been criticized as an inaccurate measure of MPB. Therefore, a realistic technique for measuring MPB was beyond the scope of our acute studies.

If in study 2, CP does not further enhance MPS compared with protein alone, it is still essential to perform study 3, because the acute effect does not always reflect the effectiveness of the supplement over time. Based on previous results (23, 44), an improvement in body composition, muscle hypertrophy and strength in the FHL is expected to be observed after 8-12 weeks of high intensity resistance training. The effects of nutritional supplementation in combination with exercise training are to be determined.

Finally, young adult male rats were used in our studies. Regardless of the findings, our results will have to be confirmed with human research if more effective nutritional supplementations are to be developed in the future. The mode of exercise in our studies was resistance exercise, and therefore the results may not be generalized to other types of exercise, such as aerobic exercise.

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Chapter II: Review of Literature

Contemporarily, the importance of muscle mass change on metabolic function, strength, and power has been well recognized. An increase in muscle mass is crucial for competitive athletic performance. The maintenance in muscle mass is essential for maintaining a healthy and productive lifestyle and reducing risk of injury. As such, the development of appropriate strategies that can accelerate muscle protein synthesis (MPS), reduce muscle breakdown and increase or maintain muscle mass is highly recommended and beneficial for multiple populations, including athletes, body builders, elderly humans with sarcopenia, and patients suffering from clinical diseases that have a high catabolic state. In order to exploit the right treatments, we must first understand the molecular mechanisms that regulate skeletal muscle protein turnover.

MOLECULAR MECHANISMS OF MUSCLE PROTEIN TURNOVER

Signaling pathways that control muscle protein synthesis

The regulation of muscle protein turnover involves interactions of gene transcription, mRNA translation, and protein degradation. Translation contains three main steps: translation initiation, elongation and termination, in which initiation is the rate limiting step in the regulation of overall MPS (5, 59). It is widely acceptable that mammalian target of rapamycin (mTOR) complex is a major regulator on MPS and muscle mass changes (166). mTOR complexes consist of mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is a central protein integrating AA, muscle contraction, and anabolic hormones (65) signaling, with activation of mTORC1-dependent signaling pathway sufficient to induce MPS and in turn muscle fiber growth and repair (72).

The core components of mTORC1 consist of mTOR, G protein β-subunit-like protein (GβL), and regulatory associated protein of mTOR (Raptor) (42, 45, 82). The activation of mTORC1 is a complex process requiring its subunits interacting with mTOR by formation, localization, and phosphorylation. mTOR is a Ser/Thr kinase containing multiple domains such as kinase domain, HEAT repeated, FKBP12-

Rapamycin Binding (FRB) domain, and regulatory domain (RD), etc (82, 193). GβL can bind to the kinase domain on mTOR to increase its kinase activity and stabilize mTOR-raptor association (82). Raptor is an adaptor protein essential for substrate binding with mTOR and thus recruiting downstream substrates to the mTOR for phosphorylation by its kinase. In addition to the core components, other molecules also interact with mTOR such as homologously enriched in brain (Rheb), Proline-rich Akt substrate 40kDa (PRAS40), and phosphatidic acid (PA) (45, 95). Of these molecules, Rheb-GTP activates mTOR kinase domain and stimulates mTOR phosphorylation at the Ser2448 site. Likewise, PA has been shown to bind to the FRB domain on mTOR, which in turn directly activate mTOR kinase activity (87, 195). Moreover, Rheb-GTP and PA are considered the only two molecules that are capable of activating mTOR signaling directly (86, 166). Conversely, PRAS40 is a protein inhibiting the activity of mTOR by directly interacting with the mTOR kinase domain and downstream substrates. In both rodents and humans, mTORC1 plays an important role during the muscle hypertrophy process in the regulation of mRNA translation initiation and elongation (14, 185). mTORC1 will be referred to as mTOR below.

Once mTOR is activated by the phosphorylation at the Ser2448 site, it further phosphorylates two other downstream factors, 70 kDa ribosomal protein S6 kinase (p70S6k) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (54, 55). p70S6k can further activate ribosomal protein S6 (rpS6), which is a component of the eukaryotic 40S ribosomal subunit (97). The activation of rpS6 results in translation of specific mRNA to increase capacity of protein synthesis. The specific mRNA contains ribosomal proteins (eIF4G), elongation factors (eEF1/2), and poly A binding proteins (156). On the other hand, 4E-BP1 mediates binding of mRNA to the 40S ribosomal subunit (56). Once dephosphorylated, active 4E-BP1 competes with eukaryotic initiation factor 4G (eIF4G) and binds to eukaryotic initiation factor 4E (eIF-4E), which causes a dissociation between eIF4E and eIF4G. In this condition, 4E-BP1-eIF-4E can still bind with mRNA but not with the 43S preinitiation complex. Conversely, hyperphosphorylation of 4E-BP1 at the Thr37/46 site dissociates itself from eIF-4E so that eIF-4E is able to bind to eIF4G and

eIF4A to start eIF4F complex formation (137, 185). This complex then becomes a bridge linking mRNA to the 43S preinitiation complex, which increases rates of mRNA translation. Both downstream pathways from mTOR ultimately increase the capability for protein synthesis.

Akt/PKB is an upstream substrate of mTOR. The phosphorylation of Akt at the Ser473 site activates mTOR predominantly via inhibiting the activity of PRAS40 and tuberous sclerosis complex 1/2 (TSC1/2) (92). TSC1/2 acts as a GTPase activating protein (GAP) converting Rheb-GTP into Rheb-GDP. Rheb-GDP is incapable of activating mTOR signals. However, the phosphorylation of TSC2 by the Akt dissociates itself from TSC1, and loses its GAP activity. Then, Rheb-GTP can activate mTOR signals. Some anabolic hormones, for instance, insulin and insulin-like growth factor (IGF)-1, can activate Akt through the phosphatidylinositol 3–kinase (PI3k) pathway (111, 112). Conversely, AMP-activated protein kinase (AMPK) α is an energy-sensing protein. During and immediately after RE, an increase in the AMP-to-ATP ratio as a result of myosin ATPase activity stimulates the phosphorylation of AMPKα at the Thr172 site (43). The activation of AMPKα reduces MPS via inhibiting mTOR and decreasing 4E-BP1 phosphorylation (16, 44).

Another step of translation initiation is the formation of the 43S preinitiation complex. This is determined by the binding of met-tRNA to the 40s ribosomal subunit with eukaryotic initiation factor 2 (eIF2)-GTP which form a ternary complex. The GTP on the complex is hydrolyzed to GDP, which releases eIF2-GDP from the 40S ribosomal subunit. eIF2α then exchanges GDP for GTP in order to participate in another round of initiation (17). This exchange process is mediated by another important initiation factor eIF2Bε. The activity of eIF2Bε is regulated by glycogen synthase kinase 3 (GSK3). GSK3 phosphorylates eIF2Bε at the Ser539 site to reduce its activity (98). Either phosphorylation of Akt or p70S6k is able to inhibit GSK-3α/β by the phosphorylation at the Ser21/9 site, leading to an activation of eIF2B (98, 102).

Regulatory systems that control muscle protein degradation

Because muscle protein gain is a sum of synthesis and breakdown, merely measuring MPS is not sufficient to interpret net protein balance. The principal proteolytic systems in skeletal muscle are classified into the ubiquitin-proteasome system, lysosomal proteolysis, and Ca^{2+}-activated proteases (i.e. calpain) (96, 143). Lysosomes are membrane-bound vesicles primarily degrading membrane proteins such as certain ligands, receptors, channels, and transporters (8). Calpain mediated proteolysis does not directly degrade the contractile proteins (i.e. actin and myosin), but it participates in the cleavage of cytoskeletal proteins such as titin, nebulin, and C-protein (145). Caspase 3 was also shown to interact with the calpain systems and be involved in actomyosin disassociation (46). Among these proteolytic systems, however, the ubiquitin-proteasome system is the primary signaling pathway that mediates myofibrillar protein degradation (96). Of this system, the proteasome complex (26S) is composed of a core subunit (20S) and two regulatory complexes (19S). The 26S subunit can only recognize and degrade ubiquitinated proteins. Three types of ligases are required for protein substrate binding with ubiqutin: the ubiquitin-activating enzyme (E1), conjugation enzymes (E2s), and specialized ligases (E3s). During the entire process of protein degradation, E3 enzyme mediated ubiquitin ligation is considered as a rate limiting step (13, 174). Two crucial ubiquitin E3 ligases in the skeletal muscle, muscle atrophy F-box (MAFbx or atrogin1) and muscle ring-finger protein 1 (MuRF1), have been shown to stimulate muscle proteolysis (4). The activation of both E3 ligases is under the regulation of transcription factors such as forkhead box O (FOXO) 3A in the nucleus (197). The phosphorylation of FOXO3A causes it to be trapped in the cytoplasm and inactive. As mentioned above, both Akt and AMPK mediate the phosphorylation of mTOR, but with opposing control over MPS. Akt is also a key factor in the phosphorylation of FOXO3A (23), whereas AMPK stimulates FOXO3A translocated into the nucleus (128). Moreover, other investigations reported an important role of NF-kB p65 induced muscle loss due to the activation of MuRF1 expression (28). Taken together, the inactivity of FOXO3A and NF-

kB p65 trapped in the cytoplasm turns off the expression of E3 ligases in the nucleus, thereby leading to a reduction of MPB.

MUSCLE ADAPTATION TO ACUTE RESISTANCE EXERCISE

Muscle protein turnover induced by an acute resistance exercise

It is commonly accepted that RE induces muscle protein accretion primarily by stimulating MPS. Fluckey et al. demonstrated in a rodent study that 16 h after 4 sessions of full extension of the lower extremity by performing squat movements, both gastrocnemius and soleus muscles showed a higher rate of protein synthesis than the muscles in non-exercised rats (59). In another research study, Hernandez et al. investigated the time course of change in MPS following RE and observed that an increase in the rate of protein synthesis in the gastrocnemius did not occur until 12 h post exercise (83), and remained elevated 24 h after exercise (83). This result is in agreement with human studies. In research by Chesley and coworkers, MPS in the biceps after performing a high-intensity elbow flexion exercise was significantly increased by about two-fold compared to the non-exercise biceps for up to 24 h (32). In another research study, this same group found that MPS induced by RE returned to baseline 36 h after exercise (117). However, Phillips et al. showed that acceleration of mixed muscle protein fractional synthesis rate (FSR) lasted up to 48 h post RE with the highest rate at 3 h, which then resulted in a significant increase in net protein balance over 48 h post exercise (141). The conflicting results about the peak period of elevated MPS are probably due to the different methodologies adopted in the MPS measurement. Chesley (32) and Macdougall (117) et al. infused L-[^{13}C]-leucine for 6 h constantly to detect MPS. In order to insure a consistent rate of appearance of endogenous energy and protein, energy intake has to be supplied 2 h before the leucine infusion. In other words, MPS was measured by L-[^{13}C]-leucine infusion when subjects were in the fed state. In contrast, MPS was determined in the Phillip et al. study (141) using a primed constant infusion of [^{2}H]-phenylalanine after an overnight fast. Moreover, Tang et al. has reported that trained individuals had a smaller change of FSR relative to that of untrained (176). Thus, having

subjects in various training statuses might be another reason for this discrepancy. Taken together, MPS can remain elevated for at least 24 h after a single bout of RE. Furthermore, skeletal muscle is composed of various proteins, including myofibrillar protein, sarcoplasmic protein, and mitochondria. These proteins may have different responses to RE; in other words, one type of protein may increase, whereas another type of protein could decrease in response to exercise. Myofibrillar protein, primarily consisting of myosin and actin, makes up more than 50% of skeletal muscle protein (139). The more myofibril packed in the muscle fibers, the greater the muscle hypertrophy, and the stronger the muscle. Therefore, the observation of changes in myofibrillar protein would be more representative to determine the muscle response to RE. Cuthbertson et al. measured rate of myofibrillar and sarcoplasmic protein synthesis over 24 h post exercise in the fed state, and found that the synthesis of both myofibrillar and sarcoplasmic proteins were increased at 6 h and 24 h after RE. These changes were proportional to the activation of Akt and p70S6k (37), which are important anabolic signaling proteins that control MPS. Overall, a single bout of RE is able to stimulate MPS, especially myofibrillar proteins.

Early research reported that acute RE did not only increase MPS, but also induced MPB, and there was a tight correlation between the changes of MPS and MPB (141). Consequently, in spite of an improvement of muscle protein accretion post exercise, net protein balance remains negative in the postabsorptive state (10, 141). MPB is commonly estimated using 3-methylhistidine (3-MH), which is found in some myofibrillar proteins like myosin and actin. Once 3-MH is excreted from those proteins, it cannot be reused. Thus, 3-MH in urine is used as an indirect marker of myofibrillar protein degradation (147, 148). To date, however, there is no direct and more reliable measuring method for MPB, because 3-MH can also be released from intestine and skin. Collectively, RE can increase both MPS and MPB. Although it stimulates muscle protein accretion, the net protein balance remains negative.

Molecular mechanisms of muscle protein turnover induced by RE

RE induced changes of muscle mass are related to changes in the rate of MPS (69, 71, 95). Numerous lines of evidence have shown that the enhancement in MPS after acute RE is largely due to translational rather than transcriptional regulation (32). For example, Chesley et al. found that an increase in MPS was positively correlated with an increase in RNA activity both at 4 h and 24 h post RE (32), whereas the change of RNA capacity was not observed. In this study, RNA activity was expressed as amount of protein synthesis per unit time and per unit RNA. RNA capacity was expressed as total RNA concentration. The data suggests that MPS elevated post exercise is related to the change in posttranscriptional events rather than transcription.

Regarding the effect of RE on signaling proteins controlling MPS, Bolster et al. (18) studied time course changes on the mTOR signaling pathway over 1 h post RE in rats. The results showed that the phosphorylation of Akt, 4E-BP1, and p70S6k peaked at 10 min after RE, and the peak phosphorylation in rpS6 occurred at 15 min after exercise. The association of eIF4E to eIF4G was also increased significantly at 10 min after exercise. The rapid response of activation of these anabolic signals during the period immediately post exercise implies a high sensitivity of muscle in response to exercise stimuli. Additionally, mTORC1 is a rapamycin-sensitive protein. Rapmycin is a bacterial product that is capable of inhibiting mTOR by associating with its intracellular receptor FKBP12. The rapamycin-FKBP12 complex can directly inhibit mTOR activity by binding the FRB domain on mTOR. Therefore, a finding that RE induced MPS was suppressed by rapamycin administration (107) further suggests that the activation of mTOR-dependent signaling pathways is necessary to promote MPS by RE. Interestingly, the study conducted by Bolster et al. did not observe changes of eIF2Bε activity and the phosphorylation of eIF2α within 1 h after RE. However, other researchers reported an important role for the activation of eIF2Bε by exercise relative to formation of the eIF4F complex for MPS during a longer period post exercise. Glover et al. found a substantial change of eIF2Bε dephosphorylation 6 h after exercise in humans (67). Earlier animal

studies also revealed that MPS was enhanced 16 h after RE due to an elevation of eIF2Bε activity (48), and not the dissociation of eIF4E from 4E-BP1 (49). Furthermore, considering the upstream regulation of the mTOR signaling pathway, it was hypothesized that the autocrine/endocrine release of IGF-1 promoted the activation of mTOR and subsequently p70S6k via the PI3k-Akt signaling pathway (70). However, the exercise-induced activation of p70S6k still occurred despite pharmacological inhibition of IGF-1 (90). Thus, the release of IGF-1 may be able to, but not be required to, activate the mTOR signaling pathway in response to an acute bout of RE. Moreover, as discussed previously, PA is an important molecule activating mTOR directly. It was recently demonstrated that mechanical loading-induced PA accumulation was necessary to stimulate mTOR activity and the elevation of PA concentration was independent of IGF-1/PI3K signaling pathway (134). Following a series of *in vitro* and *in vivo* experiments, Hornberger's laboratory found that phospholipase D (PLD) was localized at the z-line of the sarcomere, a central site for contractile force transmission (87). PLD is an enzyme synthesizing PA from phosphotidylcholine. Mechanical stimuli are capable of activating PLD, PA accumulation, and the subsequent mTOR signals. Conversely, a pharmacological inhibitor (1-butanol) of PLD blocked mechanical loading-induced PA accumulation and mTOR activation (87). The results indicate that mTOR activation in response to exercise is PLD/PA dependent. Taken together, acute RE can stimulate MPS via activating mTOR-signaling pathways. However, dissociation between the phosphorylation of signaling proteins and the change of MPS was also reported by some studies (68, 74, 125), thereby suggesting the necessity of detecting MPS directly.

During exercise, however, protein turnover is favorable for catabolic processes. As mentioned previously, AMPK is an energy sensing protein responsible for inhibiting anabolic processes that require ATP (e.g. muscle protein synthesis) and stimulating catabolic processes that generates ATP (e.g. muscle protein breakdown). During and immediately after RE, AMPK activation inhibits mTOR signals by the activation of TSC2 (93). AMPK can also directly phosphorylate mTOR at Thr2446, which inhibits the

phosphorylation at Ser2448 (31). Later, researchers suggested that AMPK activation may contribute to the inhibition of MPS during exercise and to the delayed activation of MPS during early post exercise recovery (44, 104). Nevertheless, this elevated AMPK rapidly returns to basal level after exercise, in particularly when nutrients are provided (146, 189). Beyond the changes in signaling proteins, release of catabolic hormones by RE also contributes to a negative net protein balance. Cortisol (in human)/Corticosterone (in rodent), released from the adrenal cortex, accounts for up to 95% of glucocorticoid activity (106). As we know, glucocorticoids result in rapid muscle atrophy (165), possibly by dampening MPS (122, 167) and increasing MPB (123). High intensity exercise increases plasma cortisol, which can remain elevated for at least 30 min post exercise (178, 196). Thus, this catabolic hormone causes a transient negative nitrogen balance during exercise and the period of early recovery.

In summary, both MPS and MPB are stimulated following RE, with an increase in MPS for up to 24-48 h. However, within a short period after exercise, MPS may only have a minor or no increased effect in the fasted state even though the mTOR signaling pathways may be activated. When the interval between exercise sessions is very short, athletes desire an effective muscle recovery process in order to prepare for upcoming competition. In this case, in order to shift net protein balance in favor of protein synthesis during early recovery after RE, proper nutritional supplementation post exercise may be essential for muscle protein accretion and tissue repair.

NUTRITIONAL INTERVENTION ON ACUTE RESISTANCE EXERCISE

Effect of protein ingestion on MPS

Types, amount, and timing of protein ingestion on MPS

A synergistic effect of RE and feeding on MPS during the early phase of post exercise recovery (i.e. <5 h) has been well described (11, 126, 182), and the sensitivity of MPS to nutritional stimuli can last up to 24 hours after RE (25). When it comes to nutritional supplementation post exercise, previous research provides strong evidence that

protein ingestion promotes MPS relative to exercise alone (21, 124, 175). With regards to the interaction of protein supplementation on MPS, many considerations need to be discussed, including supplement timing, the quality, and quantity of protein supplements. Recently, Burd et al. reported that the sensitizing effect of RE to protein intake could be sustained for at least 24 h (25). In this study, participants performed either low (30% of maximal strength) or high (90% of maximal strength) intensity RE to voluntary fatigue and were fed 15 g whey isolate 24 h after exercise. The results showed that the synthesis of the myofibrillar fraction was still augmented with protein feeding 24 h post RE at both exercise intensities compared to that of protein feeding at rest. Despite the synergistic effect of RE and protein supplementation on MPS anytime within 24 h after exercise, the greatest enhancement on MPS appears to be achieved when protein is provided immediately after exercise (34). This importance in protein consumption timing is illuminated by Levenhagen et al. Protein plus CHO was given either immediately (early) or 3 h (late) after a 60-min moderate intensity of cycling (114). Compared with late supplement, early protein/CHO intake elevated leg protein synthesis almost threefold after exercise, which in turn contributed to a higher net protein balance. Therefore, protein provision immediately after exercise is recommended to drive a better muscle recovery.

Our daily protein intake should be at least 0.8 g/kg for a young adult sedentary human according to the recommended daily allowance (RDA). An additional amount of protein consumption may be required depending on the quality of protein ingestion, physiological condition, and physical activity level. In order to increase MPS and/or muscle hypertrophy, the level of protein intake with exercise should maintain a positive nitrogen balance. The level of AA in the blood is a critical initiator of protein synthesis, so AA appears to stimulate MPS in a dose-dependent manner. To elucidate the response of MPS to the amount of protein intake clearly, Moore et al. examined different amounts of protein ingestion (0, 5, 10, 20, and 40 g) post RE. They observed a dose-dependent stimulation on MPS to increasing amounts of dietary protein until 20 g (equal to 8.6 g

essential AA), with MPS leveling off from 20 to 40 g protein provided post exercise (125). Simultaneously, leucine oxidation was elevated when 40 g protein was supplied compared with 20 g (125). These results indicate that 20 g high-quality protein supplementation is sufficient to maximize MPS post RE in young adults. Excessive protein consumption leads to oxidation of amino acids.

With respect to the quality of protein supplementation, whey, casein, and soy proteins are all natural proteins found in daily food. It is believed that milk ingestion (whey or casein as sources) is superior in stimulating MPS compared to a same amount of soy protein after RE (81, 190), because whey and casein contain great portions of essential amino acids (EAAs), particularly branched chain amino acids (BCAAs). Therefore, whey and casein are considered high-quality protein sources. The metabolic response of these two proteins is primarily determined by their digestive characteristics. Whey protein (WP) is acid soluble in the stomach and thus allows a rapid absorption of AA, whereas casein is converted to a solid clot in the gastric acid environment and thus digested slowly (15, 38). Therefore, WP promotes a high but short increase in whole body protein synthesis (26). Conversely, casein stimulates a low but sustained increase in protein synthesis (169). Tang et al. compared whey isolate, casein, and soy supplements on MPS after RE in young men. Whey isolate stimulated MPS 3 h post exercise to a greater extent than that given by either casein or soy protein (175). Leucine, a BCAA, is a very strong stimulator in the mTOR signaling pathway. A faster digestion and a higher portion of leucine in whey isolate appeared to account for its greater stimulation on MPS compared with the other two types of proteins. Similar results were confirmed in elderly men in a study of Burd and coworkers (26). Participants performed unilateral leg RE and then were given either 20 g casein or WP. Both rest and exercise legs showed a higher MPS during 4 h exercise recovery with WP ingestion than casein. The increased MPS was associated with a greater hyperaminoacidaemia or leucinaemia with whey supplements in this study. However, investigations comparing WP and casein do not always show consistent results. Some studies reported that both whey and casein led to a

similar myofibrillar MPS during 6 h of recovery after a high-intensity resistance exercise in young and elderly (41, 149, 181). Others even demonstrated a better effect of casein on MPS than WP (169). These conflicting results among studies are possibly due to the timing of the synthetic measurements (i.e. 1-3 h or 4-6 h). Generally, WP exerts a higher stimulatory effect on MPS during the first 3 h post exercise, but casein might have a similar effect on MPS to WP when measured over 6 h after exercise. Nevertheless, in order to pursue a faster recovery and MPS after acute RE, WP is considered best for athletes.

Possible cellular mechanisms of protein ingestion on MPS

Apparently, AA digested from protein act as precursors for muscle protein building. More importantly, proteins play an essential role in the regulation of mRNA translation initiation via augmenting the activation of mTOR signaling pathways, in particularly of leucine. Our laboratory has completed several studies in recent years addressing the effect of protein on the mTOR signaling pathway following endurance or resistance exercise. In a study by Morrison and coworkers, the phosphorylation of mTOR and p70S6k was significantly increased by a protein supplement (whey isolate = 300 mg/kg) compared to exercise alone 30 min after 3 h swimming of rats (127). In another unpublished study from our lab, rats ran on a treadmill for 50 min using a high-low interval intensity exercise protocol, and were provided a whey protein supplement (375 mg/kg) immediately after exercise. The results showed that the phosphorylation of mTOR, p70S6k, and 4E-BP1 was enhanced 45 min post endurance exercise with protein supplementation compared with exercise alone. This study also found an increased phosphorylation of FOXO3A by protein supplementation, suggesting a possible inhibitory effect of protein on MPB. Furthermore, another animal study in our lab utilized a ladder climbing protocol to simulate a resistance-type exercise, and observed an increase in the phosphorylation of mTOR, p70S6k, and rpS6 at times of 20 and 40 min post RE when whey was provided immediately after exercise compared with exercise alone (unpublished data). In contrast, AA (10g) stimulated MPS can be completely

blocked by rapamycin (40), which further indicates a central role of mTOR signal on the enhancement of MPS.

It is notable that AA/protein stimulates mTOR activation through some potential signaling pathway that is distinguished from the one stimulated by mechanical loading. High-quality of protein contains a large amount of leucine. Leucine supplementation can cause a transient increase (15-45 min after administration) in insulin secretion that could partly contribute to the activation of mTOR (3). Other evidence suggests that other upstream components through insulin-independent pathways play central roles in the regulation of AA-induced mTOR activation (161). Rag GTPases are a family of Ras small GTPase proteins, consisting of RagA, RagB, RagC, and RagD. The heterodimer RagA/B can be functional when AA stimulates GTP binding to them (161). The active heterodimer RagA/B then binds to Raptor directly and localizes mTOR to the surface of the lysosome membrane where Rheb-GTP is situated. As mentioned previously, the activation of mTOR includes formation, phosphorylation, and translocation. The translocation of mTOR to the lysosome membrane by the Rag complex facilitates its interaction with Rheb-GTP for activation (136). Moreover, vacuolar protein sorting 34 (Vps34) is considered another AA sensor. It is a novel class III PI3-kinase that can bind with Vps15 and phosphorylate phosphoinositide (PPI) to PI3P on endosomal membranes. PI3P is capable of recruiting various proteins (e.g. mTOR) to the endosome for later activation (27, 132). Byfield et al. also demonstrated that Vps34 was not stimulated by insulin, but inhibited by amino acid starvation, further suggesting that it lies on the AA-regulated pathway (27). Knock-down of Vps34, on the contrary, reduced p70S6k and activated 4E-BP1, the two downstream proteins of mTOR (27). MAP4K3 is another insulin-independent regulator of mTOR in response to AA (53). In a research study conducted by Findlay et al., overexpression of MAP4K3 increased phosphorylation of p70S6k and 4E-BP1, which actions were not affected by a PI3k inhibitor wortmannin (53). MAP4K3 may activate mTOR via the Rag complex, but further research on the link between MAP4K3 and mTOR needs to be conducted.

Taken together, exercise and AA/protein stimulate mTOR signals via divergent pathways. This could explain why combining protein supplementation with RE results in a more pronounced anabolic response than either strategy provided alone.

Effect of L-Alanylglutamine (AlaGln) ingestion on muscle protein turnover

Protein supplementation is widely used in athletes to build muscle mass. However, not all AA contribute to an anabolic response and promote muscle hypertrophy equally. It is well known that essential amino acids (EAA), especially leucine, are strong stimulators on MPS. In addition to the EAA, glutamine supplementation has been increasingly recommended for athletes in recent decades because glutamine is the most abundant free amino acid in plasma (550-750μM) and skeletal muscle (20mmol/kg wet weight muscle) (50, 66). This large concentration difference between plasma and skeletal muscle is maintained by active transporters, particularly the sodium/potassium ATPase-driven ion pump and the system A transporters (42, 151, 152, 168). Moreover, glutamine has been clearly shown to have potential benefits on glycogen resynthesis (22), protein synthesis (99, 118), prevention of muscle atrophy (159), and immune system protection (29). Glutamine also serves as a precursor in many other metabolic reactions, such as biosynthesis of nucleotides and gluconeogenesis. The source of glutamine comes from exogenous ingestion and *de novo* synthesis (130) by the body and thus it is classified as a conditionally EAA (108). Under normal conditions, the demand for glutamine can be met by synthesis within the skeletal muscle and from dietary proteins (129, 130). Under catabolic conditions such as severe illness, trauma, and overtraining, glutamine concentrations in plasma and skeletal muscle may fall below normal levels (109, 133). When the requirement for glutamine exceeds its *de novo* synthesis (76, 157), exogenous glutamine intake becomes necessary.

The effect of glutamine on muscle protein turnover

When it comes to its effect on muscle protein turnover, early research observed a positive correlation between the intramuscular concentration of glutamine and the rate of MPS (99, 118, 150). MacLennan and colleagues showed that increasing perfusate

glutamine concentration led to an increased intracellular uptake of glutamine, which in turn enhanced isolated muscle protein synthesis both in the presence and absence of insulin (118). In a human study, enteral infusion of glutamine exerted a protein anabolic effect by a detect increase in nonoxidative leucine disposal, an index of whole body protein synthesis (79). However, some other investigations failed to show a beneficial effect of glutamine on MPS (73, 188, 198). In an *in vitro* study conducted by Zhou and colleagues, it was observed that the rate of protein synthesis by glutamine administration was only seen in stressed myotubes (administered by a heat shock treatment) but not in normal-cultured myotubes (198). This is the first study indicating that the effect of glutamine on protein synthesis may be conditionally dependent. A recent study also demonstrated that the modulation of glutamine supplementation on expressions of mRNA and proteins controlling MPS and MPB was seen in diabetic rats, but not in normal non-diabetic rats (109). Moreover, Wilkinson et al. found that the addition of glutamine (0.3g/kg) to EAA plus CHO did not further enhance protein synthesis following 1.5 h of cycling (188). One possible explanation on this result is that this endurance exercise protocol was not sufficient to reduce plasma and intramuscular glutamine concentration, so exogenous glutamine was not able to affect muscle protein balance. Another possibility is that sufficient EAA were provided to maximize MPS so that the addition of glutamine would be of little benefit. This possibility was supported by an increase in leucine oxidation during exercise recovery when given EAA+CHO, suggesting a sufficient EAAs provision.

The results from the above research studies reveal that glutamine supplementation would be more essential for the maintenance of net protein balance under catabolic states such as stressful exercise and injury when the glutamine level falls. Low level of glutamine is also seen in patients with cancer, trauma, burn, surgery and those who are in intensive care (187). In these conditions, glutamine supplementation is supplied to maintain muscle glutamine content, which in turn counteracts the fall of MPS and/or inhibit MPB (77, 78).

With respect to MPB, glutamine plays an important role in the prevention of muscle atrophy. Glucocorticoid treatment results in rapid muscle atrophy both in animals and humans because of its catabolic and anti-anabolic effects on the contractile muscle proteins (165). In a study of Salehian et al., rats were administrated dexamethasone, a potent synthetic glucocorticoid (159). After 5 days of administration, rats showed significant muscle atrophy with an increase in myostatin expression in muscle. Myostatin, a member of the transforming growth factor-β superfamily, plays a role in the down-regulation of muscle mass hypertrophy. It can activate ubiquitination via increasing expression of Smad 3, Atrogin-1, and MuRF-1 (116). Myostatin also inhibits proliferation and differentiation of satellite cells to reduce the myogenic process (179, 180). Once glutamine was administered in this study, dexamethasone-induced expression of myostatin was dampened, and thereby muscle atrophy was attenuated (159). Although this study did not measure plasma or intramuscular glutamine concentration, results from previous research together with this study revealed that glucocorticoids can cause an increase in the expression of glutamine synthetase to facilitate glutamine release from the skeletal muscle for liver gluconeogenesis (76, 84, 159). The reduction of intramuscular glutamine concentration would eventually impact net protein balance in skeletal muscle. Therefore, maintenance of intramuscular glutamine concentration is not only crucial for enhancement of MPS, but also to inhibit MPB, which then prevents muscle atrophy.

Possible mechanisms of glutamine on regulations of muscle protein turnover

It was recently reported that oral administration of glutamine resulted in significant increases in plasma and muscle glutamine concentration in diabetic rats, compensating for a decreased glutamine level in such a catabolic disease (109). Nevertheless, glutamine supplementation did not further increase plasma or muscle glutamine concentration in the normal non-diabetic rats. Lambertucci et al. also reported that the modulation of glutamine supplementation on expressions of mRNA and protein controlling MPS and MPB was only seen in diabetic rats but not in normal rats. For example, glutamine supplementation increased both mRNA expression and protein

phosphorylation of Akt in diabetic rats, which triggered total protein expression of mTOR and dampened total protein expression of 4E-BP1. Furthermore, early investigations demonstrated that plasma concentration of GH can be elevated by the administration of glutamine (186), and that plasma GH was associated with skeletal MPS (60, 61). Accordingly, glutamine appears to have a potential effect on mTOR signals via increasing the GH-IGF-1 axis in catabolic states. On the other hand, research has demonstrated that activation of AMPK can increase myofibrillar protein degradation by increasing the expression of atrogin-1 and MuRF-1 in C2C12 myotubes secondary to increasing the expression of FOXO3A (128). Starving cells of glutamine was reported to cause an increase in the phosphorylated to total AMPK cellular content (199). Conversely, glutamine supplementation was found to attenuate MPB and muscle atrophy by blunting atrogin-1 and MuRF-1 expression (19, 109), indicating that glutamine may inhibit MPB via AMPK-FOXO3A pathway.

However, some other studies proposed that glutamine may promote an increase in intramuscular leucine concentration and thus indirectly activate the mTOR signaling pathway. As mentioned above, glutamine influx into skeletal muscle relies on the sodium/potassium ATPase-driven ion pump and the system A transporters (SLC1A5 and SNAT2). Once taken up into the muscle fiber (cell), glutamine becomes an efflux AA. Leucine and glutamine share the same heterodimeric solute carrier family 7 member 5 (SLC7A5)/ solute carrier family 3 member 2 (SLC3A2) bidirectional transporters. SLC7A5/SLC3A2 then exchanges intracellular glutamine for extracellular leucine for activation of the mTOR signaling pathway (109, 131). In a study by Nicklin et al., preloading cells with glutamine hastened EAA stimulated p70S6k activation as solute carrier family 1 member 5 (SLC1A5) located on the plasma membrane was able to transport glutamine into the cell initially and then allowed more EAA influx into the cell by out-flowing glutamine (131). Collectively, glutamine may activate the mTOR signaling pathway and enhance MPS, but more experiments are warranted to confirm its

direct or indirect mechanisms on muscle protein turnover. It also remains unclear whether glutamine mediates these signaling pathways following resistance exercise.

Putative benefits of L-Alanylglutamine (AlaGln)

The daily supplemental dose of glutamine recommended is approximately 20-40 g based on a 70 kg body weight in human, and no toxic side effects have been reported when large amount of glutamine was taken (168). Accordingly, exogenous glutamine supplementation is thought to be safe and well tolerated by most populations. As a nutritional supplement, however, glutamine is usually provided in the form of a capsule or as a powder. Although it can also be supplied in liquid form having a low pH that enhances palatability and reduces microbial growth (80), glutamine is also poorly soluble and unstable in such solutions (63). This limitation can be overcome by using a dipeptide form containing glutamine. Sustamine^{TM} (SUS) is such a dipeptide composed of L-glutamine and L-alanine (AlaGln). Alanine, as the second most abundant AA in plasma, is essential for liver gluconeogenesis (33, 76). However, alanine is not thought to be a key factor contributing to the regulation of muscle protein turnover (73, 159). Thus, the influence of SUS on muscle net protein balance is predominantly controlled by the portion of glutamine administration. Apparently, SUS supplementation is more stable and soluble in aqueous solution than glutamine (80). Compared with provision of glutamine alone or intact protein (wheat protein), AlaGln facilitates glutamine absorption and increases plasma glutamine concentration to a higher level (80, 153). The positive correlation between glutamine level and protein balance was also seen in some studies when glutamine-containing dipeptide was given to clinical patients (36, 154, 172). A research study found that the administration of AlaGln (containing 0.235g/kg glutamine) to post-operative patients for 3 days significantly improved whole body nitrogen balance (78). The drop of intramuscular glutamine level and MPS were also attenuated by AlaGln (78). In a study conducted by Cruzat and colleagues, AlaGln administration has also been found to attenuate muscle damage as suggested by lower inflammation biomarkers following prolonged endurance exercise (36). Taken together, AlaGln appears to have

similar or better benefits, relative to glutamine alone, on muscle net protein balance and muscle damage prevention. However, whether AlaGln stimulates MPS via mTOR signaling pathway and inhibits MPB via FOXO regulation following a RE remain to be investigated.

Effect of CHO ingestion on MPS

In order to shift muscle net protein balance in favor of accumulation, another key point is to change the catabolic hormonal environment induced by the elevation of cortisol and the suppression of insulin during and immediately after high intensity RE. As discussed before, cortisol/corticosterone can enhance MPB as well as inhibit MPS. The generation of cortisol during high intensity RE also causes muscle damage and release of glutamine from working muscles, which can further lead to a negative net protein balance (76, 84). As such, the elevation of circulating cortisol level may compensate for the anabolic effect caused by exercise and protein/AA supplementation. Lines of evidence have demonstrated that nutritional supplementation containing CHO significantly reduced circulating cortisol level during and after exercise (7, 115, 163). For example, subjects receiving CHO plus protein before and during RE showed lower muscle damage assessed by detections of myoglobin and CK compared with subjects receiving placebo (7). The attenuation of muscle damage was reported to result from a suppression of cortisol level (7, 105).

As oppose to cortisol, insulin is a strong anabolic hormone. Ingestion of certain proteins or AA can stimulate insulin release, but its secretion is highly responsive to ingestion of CHO (39, 58, 183). Insulin can act on mTOR primarily through the PI3k/Akt signaling pathway (160). Insulin first binds to the α-subunit of the insulin receptor (IR) on the extracellular side of plasma membrane, which causes auto-phosphorylation of the β-subunit of the receptor. Pleckstrin homology (PH) domain on insulin receptor substrate (IRS)-1 binds to the activated IR to trigger Src homology 2 (SH2) domains on other downstream targets such as PI3k. PI3k has two subunits and the conformational change at p85 subunit activates the catalytic subunit p110 domain. This in turn converts

phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) on the inner side of plasma membrane. PIP3 recruits the PH domain containing protein, such as PDK1, Akt, and aPKC. Akt, which then can affect the mTOR signaling pathway and thus MPS as discussed previously. Therefore, insulin may promote protein synthesis through the mTOR-signaling pathway. Due to the potential dissociation between the phosphorylation of signaling proteins and the change of MPS (68, 74, 125), direct measurement of insulin's effect on MPS was addressed.

An *in vitro* study conducted by Airhart and colleagues showed an impact of insulin on promoting MPS via increasing mRNA translation initiation (1). Also, Fluckey et al. utilized hindlimb perfusion *in situ* and demonstrated that adding insulin to the medium (containing isolated muscle) can further elevate protein synthesis in rats 16 h post RE compared to exercise alone, but non-exercised muscle is not affected by insulin (59). However, other studies reported no effect on protein synthesis by insulin after RE (20, 158). Roy et al. only observed an increased trend in fractional synthetic rate over 10 h when CHO (1g/kg) was given 0 and 1h after RE (158). In a human study conducted by Borsheim et al., CHO (100 g) provided 1 h post RE improved muscle net protein balance due to reduced MPB without affecting MPS, but the net protein balance did not reach a physiological positive value (20). This inconsistency is possibly due to the differences in insulin concentrations in those studies. For the *in vitro* study, researchers used a significantly higher insulin concentration than normal physiological level to uncover the effect of insulin on MPS. Additionally, an *in vivo* study raised local forearm insulin concentration by around 1000 fold higher than the normal physiological level and observed an elevated protein synthesis even when AA concentration was not increased (85). It indicates that increased insulin level in plasma may affect MPS when it is used in a pharmacological dose, rather than physiological dose. Yet such a high dose of insulin provision is not practical for those who desire a better response to acute or chronic exercise. Conversely, it is widely known that insulin can inhibit MPB assessed by observing a decrease in urinary 3-MH (20, 158) and this effect on MPB can be explained by an increase in the phosphorylation of FOXO3A, which then reduces the expression of

atrogin-1 (52, 74). Due to its inhibitory effect on MPB, less AA is released in both plasma and intracellular spaces. Thus, an increase in insulin concentration without AA supplementation may not stimulate protein synthesis and trigger a positive protein accretion even though anabolic signaling pathways are activated (39).

Although CHO supplementation alone fails to promote MPS after exercise, it may potentially stimulate protein synthesis in the presence of AA availability. In other words, AA stimulated protein synthesis may be amplified by the elevation of insulin level (124), and vice versa. Fujita and coworkers infused three physiological doses of insulin in one leg and demonstrated that physiological hyperinsulinemia (i.e. the postprandial dose) increased muscle blood flow which resulted in more AA delivery to the muscle (62). This increased delivery of AA, particularly leucine, is very important because both AA and insulin are able to activate mTOR signaling pathway to stimulate mRNA translation initiation, thereby contribute to an additive effect on MPS. A later research study from the same laboratory then explored the effect of EAA+CHO consumption on MPS in relation to the mTOR signaling pathway 2 h after RE (43), and found that the elevated activation of the mTOR signaling pathway by EAA+CHO is a crucial regulator in the greater MPS post RE. Miller and colleagues evaluated the independent or combined effects of an AA mixture (6 g x 2) and CHO (35 g x 2) supplement administered immediately and 1 h post RE on muscle protein metabolism (124). The results showed that the coordinated effect of AA and CHO on MPS was roughly equal to the sum of their independent effect (124). It is worth noting an issue regarding the timing response to the nutrient co-ingestion. The maximal insulin effect may not be in accordance with its peak plasma concentration, because both rate of appearance of phenylalanine from muscle into blood (reflecting MPB) and urea production were only reduced during the third hour after CHO ingestion, whereas the insulin concentration peaked ~30 min after the drink, suggesting a delayed response of insulin action. Therefore, a slowed absorbed form of AA (i.e. intact WP instead of AAs) combined with CHO ingestion may elicit a greater interactive effect on MPS.

In recent years, our laboratory has investigated the impact of the combination of protein and CHO on signaling proteins that control MPS and MPB post exercise. In a study by Morrison et al., rats swam for 3 h and were then provided a placebo (exercise alone), protein, CHO, or protein+CHO (CP) supplement (127). The phosphorylation status of mTOR related signaling proteins were measured 0, 30, and 90 min post exercise. The results showed that all the supplements increased the phosphorylation of mTOR and p70S6k after exercise, but only CP enhanced the phosphorylation of rpS6 at 30 min and 4E-BP1 both at 30 and 90 min above all other treatments. Additionally, the phosphorylation of rpS6 and 4E-BP1 was in conjunction with an increase in insulin level (127). The findings from this study suggest that CP supplement is most effective in activating the mTOR signaling pathway post exercise in comparison with CHO or protein supplement alone. Furthermore, in a human study, Ivy et al. did not only demonstrate a higher level of phosphorylation of mTOR and rpS6 by CP 45 min post exercise relative to exercise alone, but also showed that CP enhanced the phosphorylation of Akt and GSK3α/β (94). Taken together, CP, compared with CHO or protein intake individually, may drive a greater effect on MPS via promoting the activation of anabolic signaling proteins.

However, not all studies support this beneficial effect of CHO supplement on MPS. For example, Koopman and colleagues compared casein plus different doses of CHO (0, 0.15, 0.6 g/kg) on whole body protein synthesis and breakdown 6 h after RE (103). They did not observe any differences in the rate of protein synthesis, breakdown, as well as whole body protein balance among the treatments. Staples et al. then reported neither MPS nor MPB could be further affected by CHO plus WP relative to WP supplement alone during 3 h after RE (170). Also, there was no difference in leg blood flow and intramuscular signaling proteins between treatments. The discrepancy of insulin/CHO effect on MPS might be partly due to the types of protein/AA consumed, measuring timing, and doses of supplements. A maximal dose of WP (25 g) or casein (30 g) was provided in studies of Staple and Koopman, whereas Miller only provided 12 g of AA. As mentioned above, insulin could mediate an increase in MPS via increasing blood

flow. When sufficient protein is supplied (>20-25 g), however, insulin becomes permissive, but not required regardless whether it addresses more delivery of AA to the working muscle or not. In addition, in the study of Miller et al., the demonstration of a higher net phenylalanine uptake (reflecting MPS) by CHO+EAA was primarily observed during the 1st and 2nd hours post exercise compared to EAA intake alone, but the change of MPS was not observed in studies of Staple and Koopman during 3-6 h after exercise. It is still uncertain whether MPS will be augmented by adding CHO to a protein supplement within 2 h after exercise compared to protein intake alone. Also, an anabolic response is not only determined by MPS, but also by MPB. Much more work remains to be done on characterizing the role of combining CHO with protein intake on muscle protein accretion and its signaling pathways after acute RE. According to our earlier discussion, it is hypothesized that co-ingestion of CHO and protein will maximize muscle protein anabolism by increasing MPS and reducing MPB.

PROTEIN AND CHO INTERACTION WITH CHRONIC RESISTANCE EXERCISE TRAINING

Muscle adaptation in chronic resistance training

Although nutritional supplements provided immediately after acute RE plays a crucial role in MPS, the final goal is to promote muscle development over long-term resistance training. An increase in muscle mass has a strong correlation with a high muscle strength and power, which then promotes a higher quality of life and lower morbidity. Therefore, the attention to muscle hypertrophy is of great interest contemporarily. Chronic resistance training programs can primarily lead to muscle hypertrophy and secondarily stimulate myogenesis (hyperplasia). The major morphological adaptation of hypertrophy is an increase in muscle cross-sectional area (CSA), which results from both MPS and new nuclei infusion into the muscle fiber. Hornberger and Farrar showed, in a rodent study, that 8 weeks of progressive resistance training induced an increase in flexor hallucis longus (FHL) muscle mass by ~23% (89). This degree of FHL hypertrophy represented 0.3%/day accretion of muscle mass, which rate was proportional to an increase of CSA. In this study, both total and myofibrillar

proteins were increased by ~24% in exercise rats compared with non-exercise rats, suggesting that the FHL hypertrophy is mainly due to an accumulation of myofibrillar protein. In addition, skeletal muscle is composed of distinct types of fibers that differ in their capillary density, enzymes, and metabolism. The major forms of muscle fiber types are classified as type I muscle fibers (slow-twitch motor units, i.e. soleus) and type II muscle fiber (fast-twitch motor units) based on their myosin heavy chain expression. Type II fibers are further subclassified into type lla and type llb (in rodent) or IIx (in human) fibers. Gastrocnemius and plantaris contain both type II fibers, whereas FHL and extensor digitorium longus (EDL) contain mostly type IIb(x) fibers. Type II fibers can display a larger hypertrophy than type I fibers in response to RE training (120, 171). For example, a human study showed that muscle hypertrophy only occurred in type IIa and IIx fibers and not in type I fibers following 8 weeks of knee extension and leg press training (101). This phenomenon can partly be explained by the activation of p70S6k, p38 mitogen-activated protein kinase (MAPK) specifically in type II fibers post exercise (177).

Muscle protein synthesis in response to resistance training

Muscle hypertrophy is normally characterized by increasing protein accumulation in the active muscle fibers. This accumulation is due to MPS exceeding MPB over an extended period of resistance training (88). Although it is clearly understood that acute RE stimulates MPS, it may not be appropriate to explain muscle growth adaptation to chronic training only using the data obtained following acute RE. Conflicting results upon changes of MPS in response to exercise training have been reported in numerous studies. Some investigators found that chronic training attenuated acute exercise induced MPS by detecting less change of mixed MPS in trained legs than untrained legs both in human and rodent studies (47, 101, 140, 142). One possible reason for this observation could be due to the diversity of exercise intensity. For example, Phillips and coworkers observed less elevation of MPS in trained relative to untrained participants at the same absolute workload (140). This result could be expected since the adaptive muscle over the

chronic resistance training was not stressed efficiently by the same absolute workload. Another possible reason for the observation is that training may alter the extent and duration of post exercise stimulated MPS (24). This view was demonstrated by Tang and colleagues (176). Changes in fractional synthetic rate were determined 4 h and 28 h after a bout of the same relative RE intensity in untrained and trained subjects. Combining their results with previous data from the same lab (101), Tang et al. reported greater amplitude of fractional synthetic rate in the trained state at 4 h post exercise, but its rate was reduced at 16 and 28 h post exercise compared to the untrained state. Based on the results, it was speculated that training alters the window of response to acute exercise and that an increase in MPS might be missed if measurements of protein synthesis are performed at the wrong time. This view was further supported by a study conducted by Gasier et al (64), in which rats underwent a 5-week resistance training program and then MPS post exercise was measured by two different methodologies. Interestingly, the change in FSR was not observed post-exercise in trained rats compared with sedentary controls when it was measured 16 h after exercise by a traditional short-term measurement of protein synthesis (a flooding dose of ^{3}H-phenylalanine over 10-15 s at a time point of 16 h post exercise). Nevertheless, using a new methodological approach, which was capable of detecting a cumulative response of FSR over many hours (assessed over a 36-h period from 20 h before exercise till 16 h after exercise), demonstrated a significant elevation in FSR post exercise compared with sedentary controls. Collectively, these findings suggest that resistance trained individuals may have a more rapid and stronger increase in protein synthesis, but shorter duration after an acute exercise bout relative to untrained individuals.

With respect to the underlying mechanisms, it is worth noting that p70S6k has been suggested as a marker for the phenotypic changes of muscle hypertrophy. The first evidence was report by Baar and Esser, who demonstrated a tight positive correlation between p70S6k activity and the change in muscle mass after 6 weeks of training (5). The activation of p70S6k, induced by mTOR, then drives mRNA translation to increase MPS.

Conversely, Bodine et al. found that resistance training induced muscle mass change in plantaris was blocked up to ~95% when rapamycin was administered (14). This study also showed that the increase in muscle CSA was accompanied by an elevation in total amount and the phosphorylation of Akt (14). Similar results were reported in a study of Leger and coworkers that 8 weeks of training caused muscle hypertrophy relevant to a higher resting phosphorylation of Akt, mTOR, and GSK3 (113). Taken together, the elevation of MPS induced muscle hypertrophy is possibly due to the elevation of both total and phosphorylation of mTOR signaling proteins.

Activation of satellite cell on muscle adaptation

During resistance training-induced muscle hypertrophic adaptation, the control of protein translation and subsequent synthesis brings about protein accretion, while satellite cells (SCs) sometimes are required to facilitate an infusion of new myonuclei to the active muscle, thereby maintaining the myonuclear domain (quantity of cytoplasm/number of nuclei within that cytoplam) for muscle hypertrophy. SCs, underneath the basal lamina, are normally quiescent. Once activated by muscle contraction, SCs migrate into the existing muscle cell and differentiate into multinucleated myofibers for muscle hypertrophy. This crucial role of SCs was first found in a study utilizing gamma-irradiation to kill SCs (155). Thereafter, muscle size was not further increased by overload in the irradiated rats (155). Later research by Patrella et al. reported that a greater hypertrophy was driven by a greater SCs activation (138). They also indicated a ceiling size on the myonuclear domain (~2000μm^2). In other words, the myonuclei fusion by SCs is required to provide sufficient cytoplasmic space for muscle protein expansion to occur when the myonuclear domain reaches a ceiling size, below which the stimulation of MPS is probably the sole factor to induce hypertrophy. Nevertheless, McCarthy's laboratory recently pointed out that SCs were not always required for skeletal muscle hypertrophy, but were necessary for fiber regeneration (121). The results above indicate that the stimulation of MPS plays a major role during the entire process of muscle hypertrophy, while the activation of SCs is

important for a greater muscle hypertrophy as the myonuclear domain reaches critical size.

Some myogenic regulatory factors (MRF) are expressed during the proliferation and differentiation phases of myogenic cells. For example, MyoD and Myf5 expression occur during proliferation stage and determine the myogenic cell fate and formation of myoblast, while Myogenin and MRF4 function in the activation of muscle differentiation (9). These MRFs are usually considered markers for the recruitment of new nuclei, especially MyoD and Myogenin (144). The activation of SCs is also controlled by growth regulators, IGF-1 and myostatin. Typically, IGF-1s are classified into three distinct isoforms: IGF-1Ea, IGF-1Eb, and IGF-1Ec in human. IGF-1Ea and IGF-1Eb are produced primarily from the liver in response to GH secretion and then release into the circulating blood. Acute RE can increase IGF-1 in plasma and within muscle fibers (192). The increased expression of blood IGF-1 does not only activate the mTOR signaling pathway, but also mediate MRFs expression (2, 57). However, blood levels of IGF-1 do not always correlate with muscle protein accretion post exercise (119, 200). Also, due to their delayed production by liver and transportation into the muscle fibers, IGF-1Ea and IGF-1Eb may only participate in SCs differentiation and fusion rather than activation and proliferation (138). In contrast, IGF-1Ec was suggested to play a more important role than IGF-1Ea/Eb in an enhancement of muscle protein accretion and SCs activation induced by mechanical loading. So, it is considered as a Mechano Growth Factor (MGF) (70). IGF-1Ec is exclusively produced in skeletal muscle and exerts its impact by means of autocrin/paracrine action in the muscle (164). As such, IGF-1Ec can be rapidly upregulated after exercise, and subsequently stimulate MPS and the proliferation of SCs (6, 138, 194). Additionally, there is evidence showing that local IGF-1 production (IGF-1Ec) restrains FOXO nuclear localization and transcriptional activity, which then blocks its downstream proteolytic actions (162, 173). Therefore, IGF-1, especially IGF-1Ec, stimulate MPS, suppress MPB, and trigger SCs activation. On the other hand, myostatin, as discussed previously, plays a role in the down-regulation of muscle mass change.

Contrary to IGF-1, myostatin impairs muscle growth via inhibiting proliferation (179) and differentiation (110) of the SCs, which then dampen the increase in muscle mass. However, resistance loading was shown to reduce mRNA expression of myostatin with a concomitant up-regulation in MGF mRNA (100). Overall, SCs can provide a source for new myonuclei and promote greater protein accretion during resistance training.

Protein and CHO supplementations on RE training

High intensity resistance training can bring about a prolonged muscle recovery and ultimately a weaker training response if there is lack of the right nutrient intervention provided. Adequate protein ingestion combined with each exercise session is critical for muscle protein accretion. Recently, a meta-analysis performed by Cermak et al. provided evidence on the proposed effect of high quality protein supplement on the gain of muscle mass, changes of fiber-type specific CSA, and muscle strength (30). The data summarized that dietary protein supplementation (mostly whey protein) in young adults augmented the gain in fat-free mass and muscle strength after a prolonged resistance-type exercise training (30). Interestingly, a consistent result from the research included in this meta-analysis showed that milk/WP supplement can stimulate muscle hypertrophy to a greater extent than soy or CHO supplement (10, 12, 81, 184, 191). Furthermore, some other studies investigated the improvement of muscle mass change by the combination of protein and CHO intake during exercise training. For example, our laboratory recently demonstrated that a greater improvement in the body composition (calculated by the difference between the lean and fat mass) was achieved after 4.5 weeks endurance training when administering chocolate milk (protein plus CHO) relative to an isocaloric CHO supplement (51). Bird and colleagues demonstrated that CHO+EAA supplementation during and immediately following resistance training sessions increased lean body mass and muscle fiber CSA to a greater extent than CHO, EAA, or PLA over 12 weeks of training (12). With regard to the supplementation timing, Cribb and Hayes showed that a protein plus CHO and creatine supplement provided before and immediately after each workout, compared with providing the supplement in the morning

and late evening, resulted in a greater increase in type II fiber-specific CSA, lean body mass, and strength (35). This enhancement of the muscle mass appears more associated with the protein part of the supplement. Willoughby and coworkers compared the effect of protein ingestion with that of CHO intake alone on muscle development during a 10-week resistance training program. In this study, 20 g high quality protein or 20 g CHO (i.e. dextrose) were ingested 1 h before and after each daily exercise. After 10 weeks, protein supplement, relative to CHO supplement, led to a greater increase in muscle mass and strength along with a higher myofibrillar protein concentration and the mRNA expression of IGF-1 in the active muscle (191). Other researchers also found that protein ingestion could promote the activation of SCs by detecting the change in myogenin mRNA expression (75, 91, 135). Hence, it is speculated that protein ingestion provided during the period of resistance training may result in a synergistic effect on the activation of SCs, thereby a greater muscle hypertrophy would be observed than that of provided by either strategy alone.

From another point of view, elevated cortisol levels during and post exercise have enormous implications for strength athletes. The harder the resistance training is, the greater the cortisol released, and the greater protein degradation occurs in particular in type II muscle fibers (106). Thus, the anabolic benefits of exercise can actually be attenuated or negated by the elevation of cortisol and reduction of insulin levels. However, CHO supplementation can acutely and chronically reverse the changes of these two hormones and alter muscle protein turnover. A study by Tarpenning and coworkers demonstrated that CHO consumption during the 12 weeks of weight lifting exerted significant increases in both type I and type II muscle CSA, indicative of muscle hypertrophy, relative to exercise training alone (178). This study also clearly showed a high correlation between the enhancement of muscle CSA and the reduction of cortisol levels caused by CHO, suggesting that the modification of the cortisol response positively impacted muscle hypertrophic adaptation to resistance exercise training (178). Moreover, as discussed above in the study conducted by Bird et al., CHO+EAA

displayed a better muscle hypertrophy compared with CHO or EAA provided individually. This study also indicated that protein degradation assessed by measurement of 3-MH was significantly reduced by CHO+EAA relative to the PLA group and suppressed exercise-induced cortisol release (12). Therefore, co-ingestion of CHO and protein/AA may promote a greater muscle hypertrophic response via an anti-catabolic effect.

Taken together, the results from various studies suggest that CHO supplementation alone may not be able to affect muscle hypertrophy to the same extent as protein supplementation, but it may induce an anabolic hormonal environment by increasing plasma insulin and reducing plasma cortisol, thus optimize the influence of protein on muscle hypertrophy. It is clear, however, that there is a big gap in our understanding as to whether the addition of CHO to a protein supplement can enhance the effect of protein supplementation on muscle protein accretion, muscle recovery, and training adaptation during long-term resistance training.

SUMMARY

Skeletal muscle protein accretion and hypertrophy are highly complex processes regulated by various aspects, such as altered mechanical activity, hormonal control, and nutritional supplementations. Resistance exercise is a powerful stimulus for muscle protein synthesis, with long-term resistance training contributing to muscle hypertrophy. In order to speed up muscle recovery after exercise, protein supplementation post exercise takes advantage of an increase in MPS and greater activation of SCs, which allows more addition of myonuclei into myofibers. Glutamine, a conditionally EAA, potentially impacts muscle protein accretion post exercise by the activation of the mTOR signaling pathway, particularly under a catabolic state. Due to its instability and insolubility in solution, however, a dipeptide containing glutamine (e.g. AlaGln) is more favorable to ingest. Beyond that of protein or AA supplementation, the addition of CHO to a protein supplement is considered another strategy to optimize an increase in muscle mass following strenuous RE. This is possibly due to the fact that CHO ingestion might

speed the conversion of a catabolic state to an anabolic state immediately after exercise. Furthermore, CHO is capable of activating the mTOR signaling pathway as well as inhibiting protein degradation in order to assist protein supplementation in maximizing muscle mass gain. In conclusion, AlaGln supplementation or CHO co-ingestion with protein supplementation may provide means of accelerating muscle recovery and/or muscle hypertrophy following resistance exercise.

Table 2.1. The impact of an acute resistance exercise on muscle protein turnover

Subjects	Exercise protocol	supplements	Measuring time period	MPS	MPB	Net change	comments	Ref.
Young men	4 x 8 reps @ 10RM 5 x 10 reps @ 12 RM	N/A	4 h post	↑	↑	↑, but still negative	↑ AAs transport	Biolo 1995
Trained Human	4 x 6-12 reps @ 80% 1RM	N/A	4 and 24 h post	↑	N/A	N/A	↑ RNA activity without changing RNA capacity	Chesley 1992
Rats	50 times touching illuminated bar w/ 0.6g/g weight	N/A	16 h	↑	N/A	N/A		Farrell 2000
Rats	2 x 25 reps in lifting	N/A	16 h post	↑	N/A	N/A		Fluckey 1996
Rats	50 times touching illuminated bar w/ 0.6g/g weight	N/A	3, 6, 12, & 24 h post ex.	↑ @ 12 & 24 h post ex. in gas. ↑ @ 24 in soleus	N/A	N/A	↑ p70S6k activity between 6-24 h post ex. in gas.	Hernandez 2000

Table 2.1. (continued) The impact of an acute resistance exercise on muscle protein turnover

Subjects	Exercise protocol	supplements	Measuring time period	MPS	MPB	Net change	comments	Ref.
Rats	50 times touching illuminated bar w/ 0.6g/g weight	N/A	16 h	↑	N/A	N/A	Rapamycin inhibits MPS	Kubica 2005
Trained Human	4 x 6-12 reps @ 80% 1RM	N/A	36 h	<-->	N/A	N/A		MacDougall 1995
Young men	8 x 8 reps @ 80% 1RM	N/A	3, 24, and 48 h post	↑	↑ @ 3 & 24 h		%MPS>MPB	Phillips 1997

Table 2.2. The impact of glutamine on muscle protein turnover

Subjects	Special conditions	supplements	Measuring time period	MPS	MPB	Intramuscular glutamine	comments	Ref.
C2C12 myotube	TNFα for last 24 h	GLN 8mM for 5 days		N/A	↓ myostatin	N/A		Bonetto 2011
Human	RT for 6 weeks	0.65g/kg					Muscle adaptation not affected	Candow 2001
Human		Infuse GLN 0.08mmol/kg.h		↑ in gut mucosa	N/A		↓ ubiquitin mRNA	Coeffier 2003
Rats	Swim for 6 weeks	1.5g/kg.d AlaGln	21 days	N/A	N/A	↑	↓ CK, TNFα, PGE2, LDH	Cruzat 2010
Human	Postsurgical patients	24g/3h	3 h	<-->	N/A	↓ in ill <--> by GLN	A possible restriction in GLN transport in ill	Gore 2003
Patients	After abdominal surgery	GLN 0.285g/kg for 3 days		A less decrease	N/A	↑		Hammarqvist 1988
Patients	Post-operation	0.354g/kg ALA-GLN (0.235g GLN)	3 days	↑	N/A	↑		Hammarqvist 1990

Table 2.2. (continued) The impact of glutamine on muscle protein turnover

Subjects	Special conditions	supplements	Measuring time period	MPS	MPB	Intramuscular glutamine	comments	Ref.
Rats	Endotoxin, pro-deficient, low-pro+endotoxin, or 3 days starve	N/A	24-30 h after endotoxin	↓ with decreasing intramuscular GLN	N/A	↓	No relationship btwn Gly or Ala and MPS	Jepson 1988
Normal and diabetic Rats	N/A	1g/kg GLN for 15 days				↑ in diabetic rats	↑ akt, mTOR, ↓4E-BP1, atrogin 1, MuRF1	Lambertucci 2012
Isolated perfused rat hindquarter	N/A	Perfusate glutamine (0.67-5mM)	45 min of perfusion	↑ w/ or w/o insulin	N/A	↑	Positive relationship btwn intramuscular glutamine and MPS	MacLennan 1987
Isolated perfused rat hindquarter	N/A	GLN 15mM	60 min	N/A	↓	N/A	Insulin effect on MPB is less than GLN	MacLennan 1988
Rats	Swim 60 min/d for 6 weeks	1.5 g/kg ALA-GLN, or 1 g GLN	Last 21 days	N/A	N/A	↑		Rogero 2006

Table 2.2. (continued) The impact of glutamine on muscle protein turnover

Subjects	Special conditions	supplements	Measuring time period	MPS	MPB	Intramuscular glutamine	comments	Ref.
Rats	Dexamethasone 600µg/kg	GLN 20g/kg for 5 days		N/A	N/A	N/A	GLN ↓ myostatin Preserve muscle mass	Salehian 2006
Human	1.5h cycling @ 65% VO2m	CHO+EAA w/ or w/o 0.3g/kg GLN	3 h	<-->	<-->	<-->	Sufficient AAs provision	Wilkinson 2006
L8 Stressed myotubes	Heat-shock treatment	GLN 0-15mM	24 h	↑	↓ in long-lived proteins	N/A	↑ HSP70 in stressed myotube, not in the nomrla myotube.	Zhou 1997

Table 2.3. The impact of protein and/or carbohydrate on muscle protein turnover after an acute resistance exercise

Subjects	Exercise protocol	supplements	Measuring time period	MPS	MPB	Net change	comments	Ref.
Young men	4 x 8 reps @ 10RM 5 x 10 reps @ 12 RM	Infuse AAs 1h post ex.	Over 3 h after infusion	↑	N/A	N/A	AA increases MPS post ex. than at rest	Biolo 1997
Young men	4 x 8 reps @ 10RM 5 x 10 reps @ 12 RM	Infuse insulin		↑ at rest ↑ tendency post ex.	↓	N/A	Ex. ↑ BF, which does not further increase by insulin	Biolo 1999
Young human	10 x 10 reps @ 80% 1RM	EAA (0.087g/kg) 1 & 2 h post ex.	4 h post ex.	↑ @ 1-2 h after 1st drink	<-->	↑ @ 1-2 h after 1st drink		Borsheim 2002
Young human	10 x 8 reps @ 80% 1RM	CHO (100g) 1 h post ex.	3 h post drink	<-->	↓	↑ @ 3rd h, but still negative		Borsheim 2004
Young men	4 sets @ 90% or 30% to fatigue	Whey isolate (15g)	21-24 h post	↑	N/A	N/A	Sensitivity of ex. Lasts for 24h	Burd 2011
Elderly	3 x 10 reps @ 1RM	Whey protein or casein (20g)	4 h	↑ both rest and ex. by WP & casein (WP>casein)	N/A	N/A	↑ Plasma EAA to peak @ 60 min post	Burd 2012

Table 2.3. (continued) The impact of protein and/or carbohydrate on muscle protein turnover after an acute resistance exercise

Subjects	Exercise protocol	supplements	Measuring time period	MPS	MPB	Net change	comments	Ref.
Elderly human	5 x 8 reps @ 80% 1RM	caseinate 30 min before vs. after ex. Whey vs. caseinate after ex. (0.45g/kg LM)	6 h	↑ by treatments, but <--> among treatments	N/A	N/A		Dideriksen 2011
Young men	10 x 10 reps @ 70% 1RM	EAA (0.35g/kg LM) + CHO (0.5g/kg LM sucrose) 1 h post ex.	2 h post drink	↑	N/A	↑	↑ p-Akt, mTOR, p70S6K, and 4E-BP1	Dreyer 2008
Young men	N/A	Infuse AA (18g/h) And infuse insulin (5, 30, 72, 167 mU/L)	3 h	<--> among doses	↓ @ 30, 72, & 167 mU/L	<--> among doses	↑ BF by insulin	Greenhaff 2008
Elderly men	N/A	40g casein during the night	5 h	↑	<-->	↑	Prolonged increase in plasma EAA	Groen 2011

Table 2.3. (continued) The impact of protein and/or carbohydrate on muscle protein turnover after an acute resistance exercise

Subjects	Exercise protocol	supplements	Measuring time period	MPS	MPB	Net change	comments	Ref.
Young human	N/A	Raised insulin 1000 fold above basal level	4 h	↑	N/A	↑	Euglycemia	Hillier 1998
Young human	10 x 10 reps & 8 x 8 reps @ 75%	CHO (35g)+EAA (6g) 1 & 2h post	2-4h post ex.	↑ both by CP and AA	<-->	N/A	Net balance by CP is roughly equal to that of by EAA & CHO alone	Miller 2003
Young men	4 x 8-10 RM (3 sessions)	Protein (5, 10, 20, or 40g)	4 h post	↑ (40=20>10>5)	N/A	N/A	Doses of protein did not change p-S6K, rpS6, and eIF2Bε	Moore 2009
Young men	5 x 8-10 RM	Whey protein (25 g)	5 h post	↑ (in myofibril) @ 3 & 5h	N/A	N/A		Moore 2009
Young men	10 x 8 reps @ 80% 1RM	Whey protein or casein (0.3g/kg)	6 h	↑ (WP=casein over 6h)	N/A	N/A	WP>casein @ 1-3h, casein > WP @ 3-6h,	Reitelseder 2011
Human	8 x 10 reps @ 85% 1RM	CHO (1g/kg) @ 0 and 1 h post	10 h post	↑ tendency	↓	↑	↓ 3-MH, ↓ urinary urea N excretion	Roy 1997

Table 2.3. (continued) The impact of protein and/or carbohydrate on muscle protein turnover after an acute resistance exercise

Subjects	Exercise protocol	supplements	Measuring time period	MPS	MPB	Net change	comments	Ref.
Young men	4 x 8-12 RM to fatigue	whey isolate (25g) w/ or w/o CHO (50g)	3 h	↑ But CHO did not further ↑	↑	N/A		Staples 2011
Young human		Whey protein or casein (20g) 1 h post	4 h	↑ (whey = casein)	N/A	↑	Different patterns of blood amino acid responses	Tipton 2004
Young men	3 x 10 reps 4^{th} set to fatigue @ 80% 1RM	Milk or soy (18.2g) post ex.	3 h	↑ (milk > soy)	N/A	↑		Wilkinson 2007

Table 2.4. The impact of protein and/or carbohydrate on muscle mass change following chronic resistance training

Subjects	Training period	supplements	Fat free mass	Fat mass	CSA	comments	Ref.
Rats	Totally 15 sessions in 5 wks	N/A	↑	N/A	N/A	Cumulative measurement for 36 h	Gasier 2011
Rats	Progressive RE for 8 wks	N/A	↑ in FHL	N/A	↑ in FHL	↑ total and myofibrillar protein in FHL	Hornberger 2004
Young men	4 x 10 reps 8 wks	N/A	N/A	N/A	↑ in type II fibers	resting FSR: T > UT post ex. FSR: T < UT	Kim 2005
Young men	3 sessions/ wk for 12 wks	N/A	N/A	N/A	↑ in both type I & II fibers of biceps		McCall 1996
Young men	6 d/wk for 8 wks	N/A	↑ at rest, but not further ↑ post ex.	↑ at rest, but not further ↑ post ex.	N/A		Phillips 2002
Young human	Progressive RE 2d/wk for 8 wks	N/A	<-->	<-->	↑ tendency in men	Neural may stimulate increased strength during early training	Staron 1994
Young men	2-3 d/ wk for 8 wks (20 sessions totally)	N/A	N/A	N/A	↑ in both type I & II fibers	MPS in T vs. UT: ↑@ 4h ↓ @ 28 h post ex.	Tang 2008

Table 2.4. (continued) The impact of protein and/or carbohydrate on muscle mass change following chronic resistance training

Subjects	Training period	supplements	Fat free mass	Fat mass	CSA	comments	Ref.
Young men	3 x 8-10 reps @75% 1RM 2d/wk for 12 wks	EAA (6g) and/or 6% CHO solution	↑ by EAA+CHO more than other groups	<-->		↓ cortisol	Bird 2006
Young men	3d/wk for 10 wks	Protein +glucose +creatine (1g/kg) Before/after or morning/evening	↑	N/A	↑ in type II fibers	Pre-post supplement is better than morning-evening supplement	Cribb 2006
Elderly men	3 d/wk for 12 wks from 20RM to 12RM to 8RM	10g protein+7g CHO at 0 (P0) or 2h (P2) after each ex.	↑ @ P0	<-->	↑ @ P0		Esmarck 2001
Young human	60 min cycling @ 75-80% VO_2max 4.5 wks	CHO (0.94g/kg)+protein (0.31g/kg) or CHO (1.25g/kg) 0 & 1h post ex.	↑ in all groups	↓ in all groups	N/A	A larger lean and fat mass differentials by CHO+protein	Ferguson-stegall 2011
Young men	5 d/wk x 12 wks @80% 1RM	Fat-free milk (17.5g) or soy (isonitrogenous) or CHO 0 and 1 h	↑(milk>soy=CHO)	↓	↑ in both type I & II fibers		Hartman 2007
Young men	40-85% 1RM 2d/wk for 21 wks	Whey protein (15g) before and after ex.	N/A	<-->	↑ (protein =placebo)	↑ body mass	Hulmi 2009

Table 2.4. (continued) The impact of protein and/or carbohydrate on muscle mass change following chronic resistance training

Subjects	Training period	supplements	Fat free mass	Fat mass	CSA	comments	Ref.
Young men	3 d/wk for 16 wks	Protein 20g	N/A	N/A	↑	Protein ↑ SCs number	Olsen 2006
Mice	EDL overloading for 4 wks by TA Ablation	Limb exposed γ-radiation	↓	N/A	↓		Rosenblatt 1992
Young men	3 x 10 reps @75% 1RM 12 wks	6% CHO solution	<-->	<-->	↑ in both type I & II fibers	↓ cortisol level post exercise	Tarpenning 2001
Young men	4d/w for 10 weeks @ 85% 1RM	Whey+casein or CHO (20g) before and after	↑	<-->	N/A	↑ IGF-1 mRNA and myofibrillar protein	Willoughby 2007

MPS: muscle protein synthesis; MPB: muscle protein breakdown; Reps: repetitions; 1RM: one *repetition maximum; GLN: glutamine; ALA: alanine;* ↑: increase; ↓: decrease; <-->: unchanged; CSA: cross-sectional area;

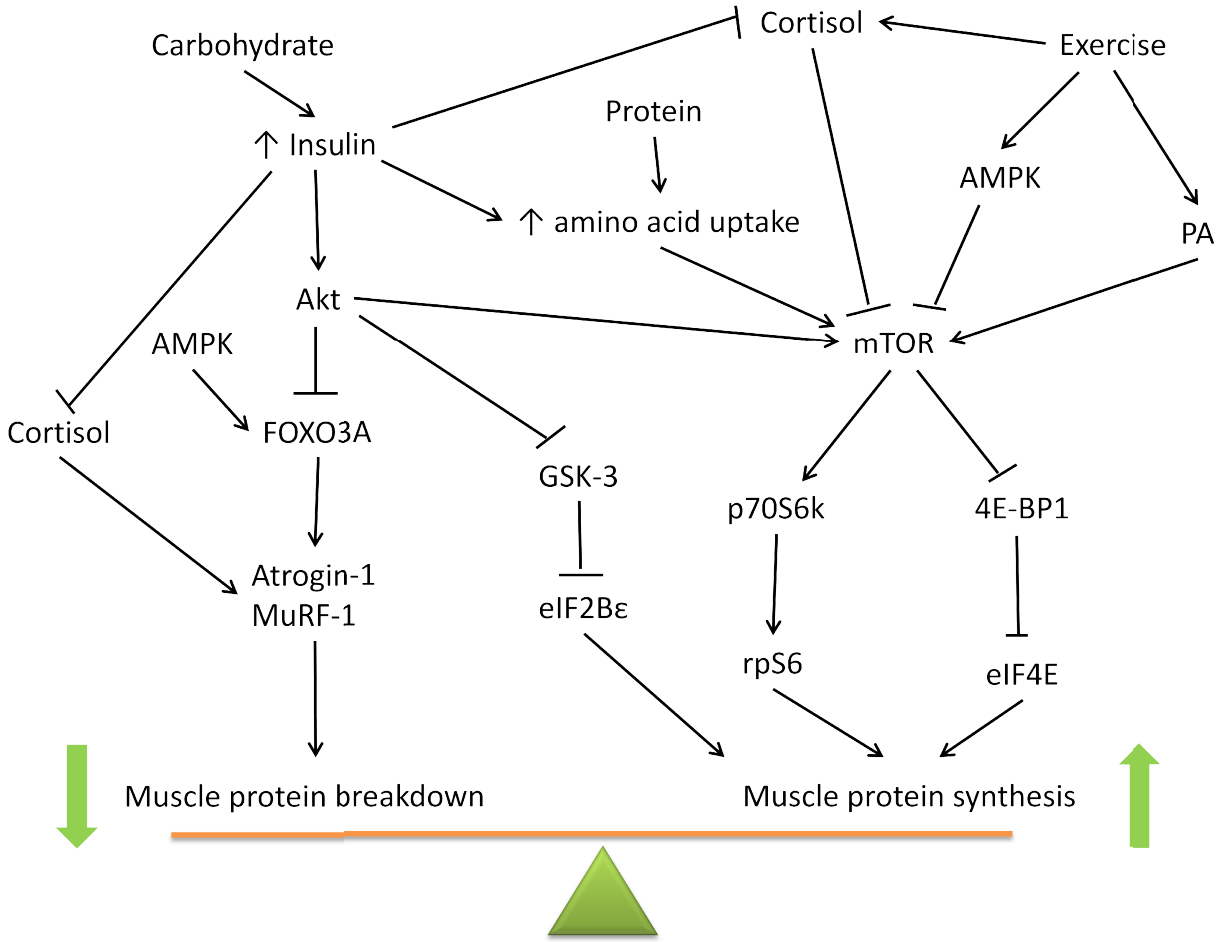

Figure 2.1. The effect of exercise, whey protein, and carbohydrate on signaling proteins that control MPS and MPB

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Chapter III: L-alanylglutamine Inhibits Signaling Proteins that Activate Protein Degradation, but Does not Affect Proteins that Activate Protein Synthesis after an Acute Resistance Exercise

ABSTRACT

Sustamine^{TM} (SUS) is a dipeptide composed of alanine and glutamine (AlaGln). Glutamine has been suggested to increase muscle protein accretion, however, the underlying molecular mechanisms of glutamine on muscle protein metabolism following resistance exercise have not been fully addressed. In the present study, two-month-old rats climbed a ladder 10 times with a weight equal to 75% of their body mass attached at the tail. Rats were then orally administered one of four solutions: placebo (PLA-glycine=0.52g/kg), whey protein (WP=0.4g/kg), low dose of SUS (LSUS=0.1g/kg), or high dose of SUS (HSUS=0.5g/kg). An additional group of sedentary (SED) rats were intubated with glycine (0.52g/kg) at the same time as the ladder-climbing rats. Blood samples were collected immediately after exercise, and either 20 or 40 min after recovery. The flexor hallucis longus (FHL), a muscle used for climbing, was excised at 20 or 40 min post exercise and analyzed for proteins regulating protein synthesis and degradation. All supplements elevated the phosphorylation of FOXO3A above SED at 20 min post exercise, but only the SUS supplements significantly reduced the phosphorylation of AMPK and NF-kB p65. SUS supplements had no effect on mTOR signaling, but WP supplementation yielded a greater phosphorylation of mTOR, p70S6k, and rpS6 compared with PLA at 20 min post exercise. However, by 40 min post exercise phosphorylation of mTOR and rpS6 in PLA had risen to levels not different than WP. These results suggest that SUS blocks the activation of intracellular signals for MPB, whereas WP accelerates mRNA translation.

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INTRODUCTION

Glutamine is the most abundant amino acid in plasma (550-750 μM) and skeletal muscle (20 mmol/kg wet weight muscle) in humans (16, 23). Under normal conditions, the demand for glutamine can be met by synthesis within the skeletal muscle and from dietary proteins (39, 40). Under catabolic conditions such as severe illness, trauma, and overtraining, glutamine concentrations in plasma and skeletal muscle may fall below normal levels (33, 41). When the requirement for glutamine exceeds its *de novo* synthesis, exogenous glutamine intake becomes necessary. Thus, glutamine is classified as a conditionally essential amino acid (31). It was found that the intramuscular glutamine concentration was highly associated with the rate of muscle protein synthesis (MPS) in isolated perfused muscle (34). Therefore, maintaining or increasing glutamine concentration in skeletal muscle appears crucial for MPS, particularly under a catabolic state. In the past two decades, glutamine supplementation has been used by athletes because it has been reported to increase glycogen synthesis (12) and protein synthesis (30, 34), and prevent muscle atrophy (47). A dipeptide containing glutamine also was reported to improve cycling exercise performance by increasing time to exhaustion (26).

MPS is primarily controlled by the phosphorylation of proteins in the mammalian target of rapamycin (mTOR) signaling pathway. mTOR is an important kinase that integrates upstream signals from muscle contraction, amino acids, and growth factors to stimulate MPS via mediating mRNA translation initiation. Once mTOR is activated by phosphorylation at its Ser2448 site, it further phosphorylates 70-kDa ribosomal S6 protein kinase (p70S6k) (17, 18). p70S6k activates Ribosomal protein S6 (rpS6), which results in translation of mRNA to increase capacity of protein synthesis (29). Conversely, the main suppressors limiting muscle protein breakdown (MPB) are the muscle ubiquitin E3 ligases. Expression of these ligases is regulated via the forkhead box O-3A (FOXO3A) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) dependent signaling pathways (14, 38, 50). Protein intake can elicit MPS via the activation of the mTOR signaling pathway at rest or after exercise (4, 36). However, there is limited information on the effect of glutamine on signaling pathways that control protein synthesis and degradation. A recent research study suggested that glutamine alleviates the loss of muscle mass in diabetic rats via the activation of the mTOR signaling pathway as well as the

inhibition of ubiquitin E3 ligases expression (33). However, it remains unclear whether glutamine mediates these signaling pathways in conditions of resistance exercise.

As a nutritional supplement, L-Gln is usually provided in the form of a capsule or as a powder. It can also be supplied in liquid form, but requires the liquid be of low pH to enhance palatability and reduce microbial growth. However, due to low solubility and stability of glutamine in low pH solutions (3, 22), glutamine dipeptides such as L-alanylglutamine (AlaGln) have been designed to improve upon these physical limitations in order for glutamine to be used in sports drinks and health products (25, 44). Compared with glutamine or wheat protein, AlaGln facilitates glutamine absorption and increases plasma glutamine concentration to a higher level (25, 44). AlaGln administration has also been found to attenuate muscle damage as suggested by lower inflammation biomarkers following prolonged endurance exercise (15). In the present study, we investigated the effects of acute Sustamine^{TM} (SUS) supplementation, a dipeptide composed of alanine and glutamine, on the signaling pathways controlling MPS and MPB post resistance exercise. Comparisons were made relative to whey protein (WP) supplementation.

MATERIALS AND METHODS

Animals A total of eighty-nine male Sprague-Dawley rats were obtained at approximately 2 to 3 months of age from Charles River (Wilmington, MA). Rats were housed 2 per cage and provided standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21° C. A reverse artificial 12 h dark-light cycle was set with the light phase from 8:00 pm to 8:00 am. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conform to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Exercise familiarization Following 1 week of acclimation to their new environment, each rat underwent 3 repeated sessions of ladder climbing separated by 1 day between each session to familiarize them with the exercise protocol. During the familiarization period, the rats carried no weights. The rats climbed a ladder 1 meter in height on an incline of 85° with 2 cm grid rungs 8 times each practice session with a 2-minute rest between climbs. The rats also completed 3

practice sessions of climbing separated by 1 day between each session with 50, 60 and 70% of their body mass attached to their tails, respectively. The weight was attached at the base of the tail with foam tape (3M Conan) and a Velcro strap. Rats were encouraged to climb by lightly tapping their tails with a bottle brush.

Experimental protocol On the experimental day, following a two-hour fast, rats climbed the ladder 10 times with a weight equal to 75% of their body mass attached at the base of the tail. There was a 2-minute rest period between each climb. Once the exercise protocol was completed, a 0.7 ml blood sample was collected from the tip of the tail. Whey protein (WP=0.4g/kg), low-Sustamine^{TM} (LSUS=0.1g/kg, L-alanylglutamine (AlaGln); Kyowa Hakko Bio. Ltd., New York, NY), high-Sustamine^{TM} (HSUS=0.5g/kg), or placebo (PLA=0.52g/kg glycine, isonitrogenous to the HSUS), was given in randomized order immediately post resistance exercise by intubation. Sixteen rats were used as sedentary controls and received an intubation of glycine (SED: glycine=0.52g/kg) at the same time. Rats in each treatment group were subdivided by time of euthanasia, which occurred at 20 or 40 minutes post intubation (n = 8-13 per group). A second blood sample was collected immediately prior to euthanasia. Following the second blood sample, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg of body weight) at which time the flexor hallucis longus (FHL) muscles were excised, freeze clamped in liquid nitrogen and stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg of body weight).

Blood Analysis From each blood sample, 0.1 ml was withdrawn and immediately transferred to a test tube containing 10% perchloric acid (PCA). All blood samples and the PCA tubes were centrifuged at 3,000 g for 10 min at 4ºC with a FS-20 microtube rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). After centrifugation, the cleared plasma samples were transferred to several test tubes and stored at -80°C for later analysis of glucose, insulin, growth hormone (GH) and IGF-1. The PCA extracts were analyzed for lactate. Plasma glucose was determined using a colorimetric method, which employs glucose oxidase and a modified Trinder color reaction (Trinder, 1969). Plasma insulin was measured using a

radioimmunoassay kit (Millipore Corporation, MA) with coefficient of variation (CV) <10%. Plasma GH was measured using a sandwich ELISA kit with CV<8% (Millipore Corporation, MA). The quantification was visualized at dual wavelengths of 450nm and 630nm. Plasma IGF-1 was determined using a high-sensitivity ELISA kit with CV<10% (Immunodiagnostic Systems Inc., AZ) and read at dual wavelengths of 450nm and 630nm. Lactate was measured according to Hohorst (1965).

Immunoblot analysis Immunoblot analysis was performed as previously described (7). In brief, ~80 mg of muscle was homogenized in ice-cold homogenization buffer (20 mM HEPES, 2 mM EGTA, 50 mM (sodium fluoride) NaF, 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DL-dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 mM sodium orthovandate (Na_2VO_4)) at a 1:8 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). The homogenates were centrifuged at 14,000 g for 10 minutes at 4°C, and the supernatants were taken for measurement of total protein concentration and of the phosphorylation of designated cell signaling proteins by immunoblotting. All muscle homogenate aliquots were then stored at -80 ºC until analysis.

Muscle samples (60μg) were combined with an equal amount of Laemmli sample buffer (125mM Tris, 20% glycerol, 20% SDS, 0.25% bromophenol blue, and β-mercaptoethanol, pH 6.8) and boiled at 95 ºC for 10 min in order to denature muscle proteins (32). Then, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% resolving gel at 130 V for 90 min (Bio-Rad Laboratories, Hercules, CA.). The resolved proteins were then electrically transferred onto a nitrocellulose membrane (pore size: 0.45 μm; GE Healthcare Life Sciences, Pittsburgh, PA.) using a wet transfer unit (Bio-Rad Laboratories, Hercules, CA.) at 90 V for 90 min. Ponceau S. (0.1% in 0.5% acetic acid) was used to verify the completeness of the transfer. The membranes were then washed in Tris-buffered saline (TBS) with 0.06% Tween20 (TTBS) to remove the Ponceau S. staining, and then the membranes were blocked in 7% nonfat milk in TTBS (blocking buffer) for 1 h at room temperature (RT). The membranes were then incubated with the appropriate primary antibody overnight at 4 ºC. The

targeted phosphorylated proteins were mTOR (Ser2448), p70S6k (Thr389), rpS6 (Ser235/236), protein kinase B (Akt) (Ser473), 5' adenosine monophosphate -activated protein kinase (AMPK) (Thr172), FOXO3A (Ser318/321), and NF-kB p65 (Ser536). Alpha-tubulin (α-tubulin) was used as an internal loading control. All the antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). Following overnight primary antibody probing, all membranes were washed 5 min, 3 times with TTBS. Then, the membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (Cell Signaling Technology, Beverly, MA). After 3 additional 5 min washes, the membranes were visualized by enhanced chemi-luminescence (ECL) in accordance to the manufacture's instructions (Perkin Elmer, Boston, MA). All membranes were stripped and re-probed for α-tubulin as an internal loading control. All western blots were performed in duplicate for each muscle sample to ensure reproducibility (CV<8%). Images were captured using a charge-coupled device camera in a ChemiDoc system (Bio-Rad, Hercules, CA). Intensity of each band was quantified with Quantity One analysis software (Bio-Rad) and expressed as a percentage of a standard.

Statistical analysis A two-way analysis of variance (ANOVA) was performed to determine significant treatment, time and, treatment by time effects for all blood parameters. When there was a significant F test identified, differences among means were determined using Fisher's least significance difference (LSD) post hoc analysis. A one-way ANOVA was used for analyzing the data obtained from western blots, and Fisher's LSD post hoc test was performed to compare mean differences among treatments. Differences with P-values < 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics v19.0 software (IBM Corporation, Armonk), and all data were expressed as mean ± standard error of the mean (SEM).

RESULTS

Blood lactate, glucose, insulin, GH, and IGF-1 level

Blood lactate was significantly elevated immediately post exercise ($p<0.05$), and then declined to basal level at 20 and 40 min post exercise. Blood lactate did not differ across treatments at 20 or 40 min post exercise ($p>0.05$) (Table 3.1.). Plasma glucose was not different

among groups immediately post exercise ($p>0.05$). However, plasma glucose was significantly reduced in the WP group both at 20 and 40 min post exercise. At 40 min post exercise, plasma glucose in WP was also significantly lower than that of the SED group ($p<0.05$) (Table 3.1.). Plasma insulin was transiently increased in SED, PLA and WP groups at 20 min post exercise ($p<0.05$), and reduced in the LSUS group (Table 3.1.). However, by 40 min post exercise insulin levels had returned to immediate post-exercise levels ($p>0.05$). Plasma GH was significantly reduced by exercise ($p<0.05$), but was not different than SED by 20 min post exercise. However, 40 min post exercise GH was elevated in the HSUS group ($p<0.05$) above all other treatment groups ($p<0.05$) (Table 3.1.). There was no difference in plasma IGF-1 across treatments or time (Table 3.1.).

The signaling proteins that control muscle protein breakdown

FOXO3A phosphorylation for WP, LSUS and HSUS was significantly increased above SED at 20 min post exercise ($p<0.05$) (Figure 3.1.A). At 40 min post exercise, there was no difference across treatment groups ($p>0.05$) (Figure 3.1.B). The phosphorylation of AMPK and NFkB p65 were significantly inhibited by LSUS and HSUS at 20 min post exercise compared with SED and PLA ($p<0.05$). These effects were not seen at 40 min post exercise (Figure 3.2., 3.3.). Akt is an upstream substrate of FOXO3A, but the phosphorylation of Akt did not show the same pattern as FOXO3A. Akt phosphorylation was significantly reduced in the WP, LSUS and HSUS groups compared with the SED group 20 min post exercise. This reduction in Akt phosphorylation was maintained within the WP and LSUS groups at 40 min post exercise ($p<0.05$) (Figure 3.4.).

The signaling proteins that control muscle protein synthesis

At 20 min post exercise, the phosphorylation of mTOR was significantly elevated in WP ($p<0.05$), but not increased in PLA. However, PLA demonstrated a significantly greater mTOR phosphorylation state relative to the SED at 40 min post exercise. Also, at 40 min post exercise mTOR phosphorylation was significantly increased above SED in WP and HSUS (Figure 3.5.). Phosphorylation of p70S6k was not increased in PLA at either 20 or 40 min post exercise.

However, WP significantly increased the phosphorylation of p70S6k at both 20 and 40 min post exercise above SED and PLA ($p<0.05$). p70S6k was also activated by LSUS and HSUS above the SED at 40 min post exercise (Figure 3.6.). Although rpS6 is a downstream target of p70S6k, phosphorylation of rpS6 both at 20 and 40 min post exercise was not enhanced above PLA for LSUS or HSUS. However, WP was able to elicit a greater phosphorylation of rpS6 PLA 20 min post exercise ($p<0.05$) (Figure 3.7.).

DISCUSSION

Glutamine, a conditionally essential amino acid, has been shown to play an important role in the regulation of muscle protein turnover under various conditions (30, 34, 56). When compared with glutamine, AlaGln ingestion produces a higher and more sustained plasma glutamine concentration (Harris et al. 2012). AlaGln has also been found to be more stable in solution than glutamine (3, 22). Positive correlations between plasma glutamine level and protein balance have also been seen in studies when a glutamine-containing dipeptide was given to clinical patients (15, 45, 49). However, to our knowledge, the present investigation provides the first insight into the phosphorylation states of enzymes controlling MPS and MPB in response to SUS supplementation after resistance exercise.

The primary findings of the present study were that SUS altered, immediately post exercise, the phosphorylation state of signaling proteins in a manner that theoretically should reduce MPB, while WP accelerated the phosphorylation of proteins in the mTOR-dependent signaling pathway thereby theoretically activating MPS. With respect to the signaling proteins that control MPB, the principal proteolytic systems in skeletal muscle are classified into the ubiquitin-proteasome system, lysosomal proteolysis, and Ca^{2+}-activated proteases (i.e. calpain) (28, 43). Among these proteolytic systems, the ubiquitin-proteasome system is the primary signaling pathway that mediates myofibrillar protein degradation (28). Two crucial ubiquitin E3 ligases in the skeletal muscle, muscle atrophy F-box (MAFbx or atrogin-1) and muscle ring-finger protein 1 (MuRF-1), have been shown to stimulate muscle proteolysis (5). The activation of these ubiquitin E3 ligases is under the regulation of FOXO3A, a critical transcription factor in the nucleus (55). In the present study, WP and two doses of SUS yielded a transient increase in

the phosphorylation of FOXO3A above the sedentary level at 20 min post exercise. Phosphorylated FOXO3A can be exported into the cytoplasm where it becomes inactive, and thereby reducing the expression of the ubiquitin E3 ligases in the nucleus. Akt, which is activated by insulin/IGF-1 via the phosphatidylinositol 3-kinase (PI3k) pathway, is considered an important regulator of FOXO3A phosphorylation (13). Nevertheless, we found that the phosphorylation of Akt was significantly reduced at 20 min post exercise when WP, LSUS and HSUS supplements were provided and at 40 min post exercise. Therefore, it seems unlikely that the insulin-Akt pathway was involved in the phosphorylation of FOXO3A in the present study. Rather, we found that the phosphorylation of AMPK was significantly reduced by both doses of SUS at 20 min post exercise, which was inversely related to the phosphorylation of FOXO3A. AMPK, an important energy sensor, is another upstream regulator of FOXO3A. Research has demonstrated that activation of AMPK can increase myofibrillar protein degradation by increasing the expression of atrogin-1 and MuRF-1 in C2C12 myotubes secondary to increasing the expression of FOXO3A (38). Starving cells of glutamine results in an increase in the phosphorylated to total AMPK cellular content (57). Moreover, glutamine supplementation has been found to attenuate MPB and muscle atrophy by blunting atrogin-1 and MuRF-1 expression (10, 33). Therefore, it is reasonable to assume that the phosphorylation of FOXO3A following SUS supplementation was mediated by the inhibition of AMPK, which in turn would likely reduce expression of atrogin-1 and MuRF-1 and prevent MPB after resistance exercise.

It is noteworthy that the phosphorylation of NF-kB p65 was also transiently reduced by both doses of SUS at 20 min post exercise relative to the placebo and WP groups. Cai et al. reported that the activation of the NF-kB p65 signaling pathway leading to muscle loss was due to the activation of MuRF-1 (Cai et al. 2004). Accordingly, the inhibition of NF-kB p65 phosphorylation by SUS might be another way of exerting an inhibitory effect on MPB post exercise. Interestingly, the phosphorylation states of AMPK and NF-kB p65 were not influenced by the WP supplement. Previous research suggests that protein ingestion can promote MPS without changing MPB relative to exercise alone (11, 35, 51), and our results support this position. Taken together, we suggest that SUS supplementation post exercise will inhibit MPB

via suppressing the activation of AMPK-FOXO3A and NF-kB p65, whereas WP has a negligible effect on the signaling pathways that control MPB.

With respect to the signaling proteins that control MPS, the activation of mTOR stimulates the phosphorylation of p70S6k (19), which then phosphorylates its downstream substrate protein rpS6 resulting in translation of mRNA to increase the capacity for protein synthesis (29). In the current investigation, WP provided immediately after resistance exercise yielded a greater increase in the phosphorylation states of mTOR, p70S6k, and rpS6 relative to the placebo and sedentary groups at 20 min post exercise. These results are in concert with a previous study from our laboratory demonstrating that provision of whey isolate after endurance exercise transiently enhanced the phosphorylation of mTOR and p70S6k in the quadriceps muscles of rats (37). Similar results have also been found in human subjects when they were supplemented with whey protein post resistance exercise (27).

When no supplement was provided, an increased phosphorylation of mTOR was delayed until 40 min post exercise. Interestingly, exercise alone resulted in the phosphorylation of rpS6 at both 20 and 40 min post exercise. However, the phosphorylation of p70S6k was not affected. In agreement with these results, Bolster et al. found an increase in rpS6 phosphorylation in response to resistance exercise without changing the phosphorylation status of p70S6k (9). Aside from being phosphorylated by p70S6k, rpS6 can be directly phosphorylated by 90-kDa ribosomal S6 kinase ($p90^{RSK}$), a component of the extracellular-signal-regulated kinase (ERK) 1/2 cascade (42, 46). Therefore, activation of ERK 1/2 by muscle contraction may explain how exercise alone activated rpS6 post exercise without affecting the phosphorylation of p70S6k.

Although MPS was not directly measured in the present study, protein/AAs supplementation has been clearly shown to stimulate MPS both at rest (4) and after different types of exercise (2, 11, 36, 52). This increase in protein synthesis has been correlated with the activation of the mTOR signaling pathway and modulation of its downstream target proteins involved in mRNA translation initiation (8, 24). Therefore, our results indicate that WP supplementation soon after exercise is capable of hastening the activation of the mTOR signaling pathway and muscle protein synthesis. Akt is an upstream activator of mTOR (48) and is

activated by insulin via the PI-3 kinase pathway. However, we found that although WP transiently increased the plasma insulin level, the phosphorylation of Akt was significantly decreased below the SED level. Accordingly, the activation of the mTOR signaling pathway by the WP supplement does not appear to have resulted from the activation insulin-PI3k-Akt pathway. Moreover, neither exercise nor WP influenced the plasma IGF-1 level suggesting this hormone also was not related to the phosphorylation of mTOR and its downstream target proteins. It is likely that the influence exerted over the mTOR pathway by the WP supplement was due to leucine activation of mTOR. Leucine is a strong independent activator of mTOR and a prominent amino acid in whey (1, 2, 9).

It was of interest to note that both doses of SUS increased p70S6k phosphorylation relative to the SED at 40 min post exercise while p70S6k phosphorylation for placebo was not. The activation of p70S6k has been suggested to correlate with muscle hypertrophy (6), and therefore suggests that SUS may potentially impact MPS through activating p70S6k. Plasma GH level dampened immediately after exercise was elevated above the basal level by HSUS at 40 min post exercise. Early investigations demonstrated that plasma concentration of GH can be elevated by the administration of glutamine (53), and that plasma GH was associated with skeletal MPS (20, 21). However, a recent study reported no effect of systemic concentration of anabolic hormones on MPS and phosphorylation of signaling proteins (54), but suggested local anabolic hormones may predominate MPS post exercise. Moreover, the present study showed that phosphorylation states of mTOR and rpS6 were not influenced by SUS at either 20 or 40 min. Although SUS increased the phosphorylation of p70S6k, it appears that SUS only has a slight impact on the mTOR signaling pathway. A recent study demonstrated that the modulation of glutamine supplementation on expressions of mRNA and proteins controlling MPS and MPB was seen in diabetic rats but not in normal non-diabetic rats (33). In an *in vitro* study conducted by Zhou and colleagues, it was observed that the rate of protein synthesis after glutamine administration was only seen in stressed myotube (administered by a heat shock treatment) but not in normal-cultured myotube (56) indicating that the effect of glutamine on protein synthesis may be conditionally dependent. Perhaps our SUS supplementation would be more effective in stimulating MPS under states of stress when catabolism is high such as following muscle injury,

severe disease or burns, or over training when glutamine level falls below its normal physiological level.

In conclusion, the results of the current study provide indirect evidence that SUS inhibits MPB via suppressing activation of AMPK-FOXO3A and NF-kB p65 post exercise, whereas WP appears to accelerate the activation of the mTOR signaling pathway and promote MPS. The actions of WP on the phosphorylation of mTOR does not appear to be hormonally controlled, but was most likely due to the actions of leucine, a prominent amino acid in whey. Together, these findings suggest that a combination of WP and SUS supplementation post exercise might result in the phosphorylation of metabolic regulatory enzymes in a manner that would increase MPS and decrease MPB, thereby maximizing muscle protein accretion.

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Table 3.1. Blood lactate, plasma glucose, insulin, GH, and IGF-1 concentration at 0, 20, and 40 min post exercise

	Treatment				
	SED	PLA	WP	LSUS	HSUS
Lactate (mM)					
0 min	1.21±0.12	3.17±0.27 *	2.61±0.21 *	3.00±0.24 *	2.63±0.14 *
20 min	1.14±0.25	1.28±0.10 †	1.35±0.22 †	1.2±0.18 †	1.59±0.20 †
40 min	1.40±0.14	1.39±0.10 †	1.28±0.11 †	1.26±0.15 †	1.42±0.09 †
Glucose (mM)					
0 min	6.21±0.29	6.67±0.25	6.46±0.24	6.37±0.27	6.32±0.23
20 min	5.38±0.36	5.97±0.43	5.14±0.18 †	5.75±0.37	5.93±0.31
40 min	6.47±0.21	5.68±0.20 †	5.43±0.26 † *	5.89±0.28	6.13±0.12
Insulin (pM)					
0 min	339.39±38.38	276.81±30.13	292.21±33.48	262.05±24.34	276.46±36.21
20 min	498.22±113.93	394.8±57.25 †	418.69±68.80 †	220.13±30.14 *#§	356.53±41.53
40 min	364.44±35.55	349.58±44.20	237.05±30.44 #*f*	215.56±27.79 #	281.91±26.95
GH (ng/ml)					
0 min	14.83±4.01	3.31±0.68 *	2.64±0.44 *	2.49±1.00 *	4.53±1.96 *
20 min	9.62±4.57	9.15±4.68	12.89±8.96	2.78±0.91	9.75±5.43
40 min	5.06±3.20	9.06±4.15	10.56±6.57	2.98±1.51	19.69±7.01 †*# ¶
IGF-1 (ng/ml)					
0 min	1071.88±56.5	1017.32±36.6	1073.92±40.00	1062.72±33.72	1045.30±40.84
20 min	958.35±91.39	988.07±55.31	1137.03±77.58	1113.23±92.12	1065.11±97.86
40 min	1093.95±70.89	1071.91±59.53	1127.24±69.67	1094.8±48.12	1111.97±48.66

Data are presented as mean + SEM (n=6-13 per group). †, $p<0.05$ vs. 0 min in the same treatment. *f*, $p<0.05$ vs. 20 min in the same treatment. *, $p<0.05$ vs. SED in the same time point. #, $p<0.05$ vs. PLA in the same time point. §, $p<0.05$ vs. WP in the same time point. ¶, $p<0.05$ vs. LSUS in the same time point.

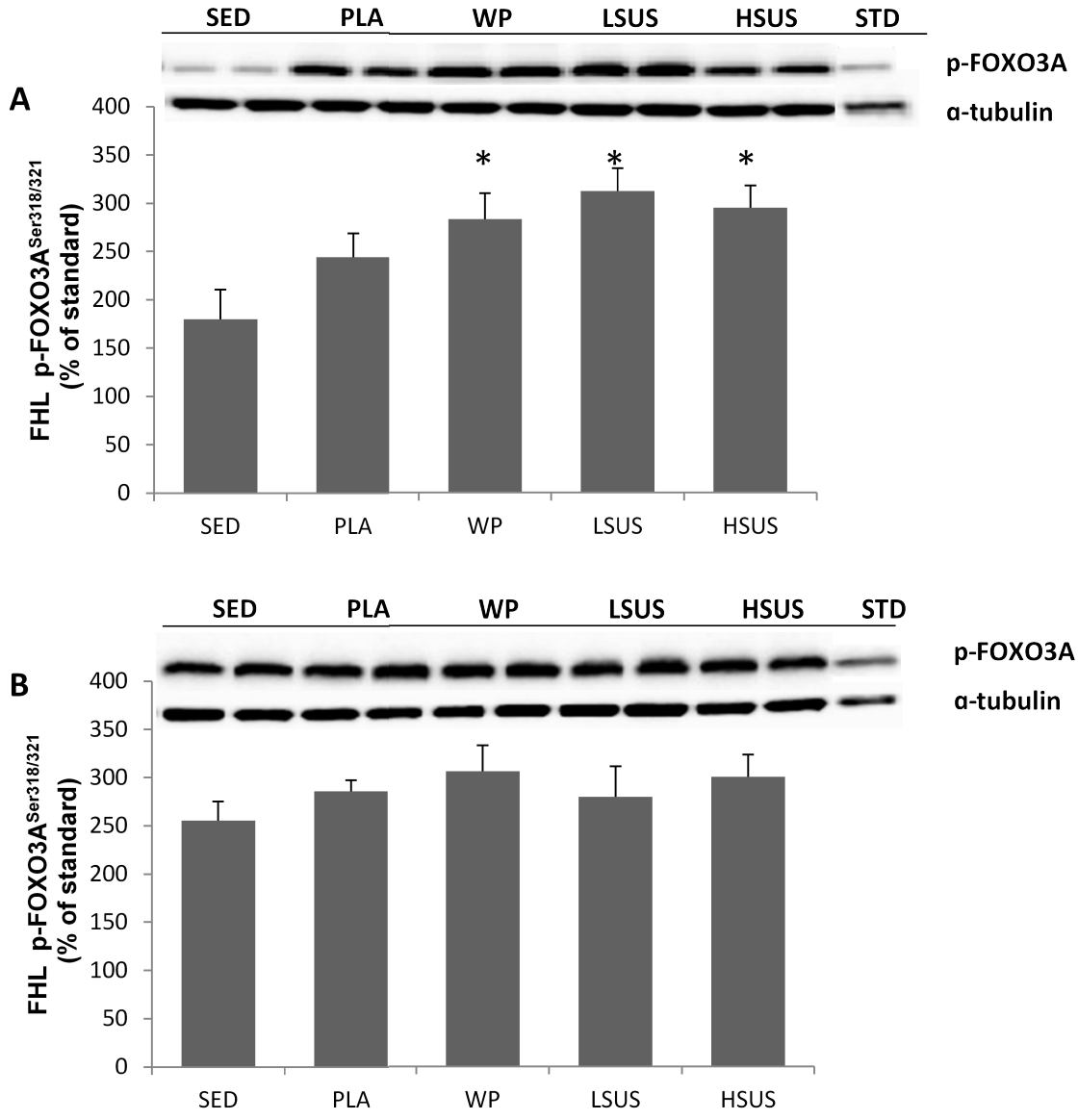

Figure 3.1. FOXO3A phosphorylation at $Ser^{318/321}$ expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED.

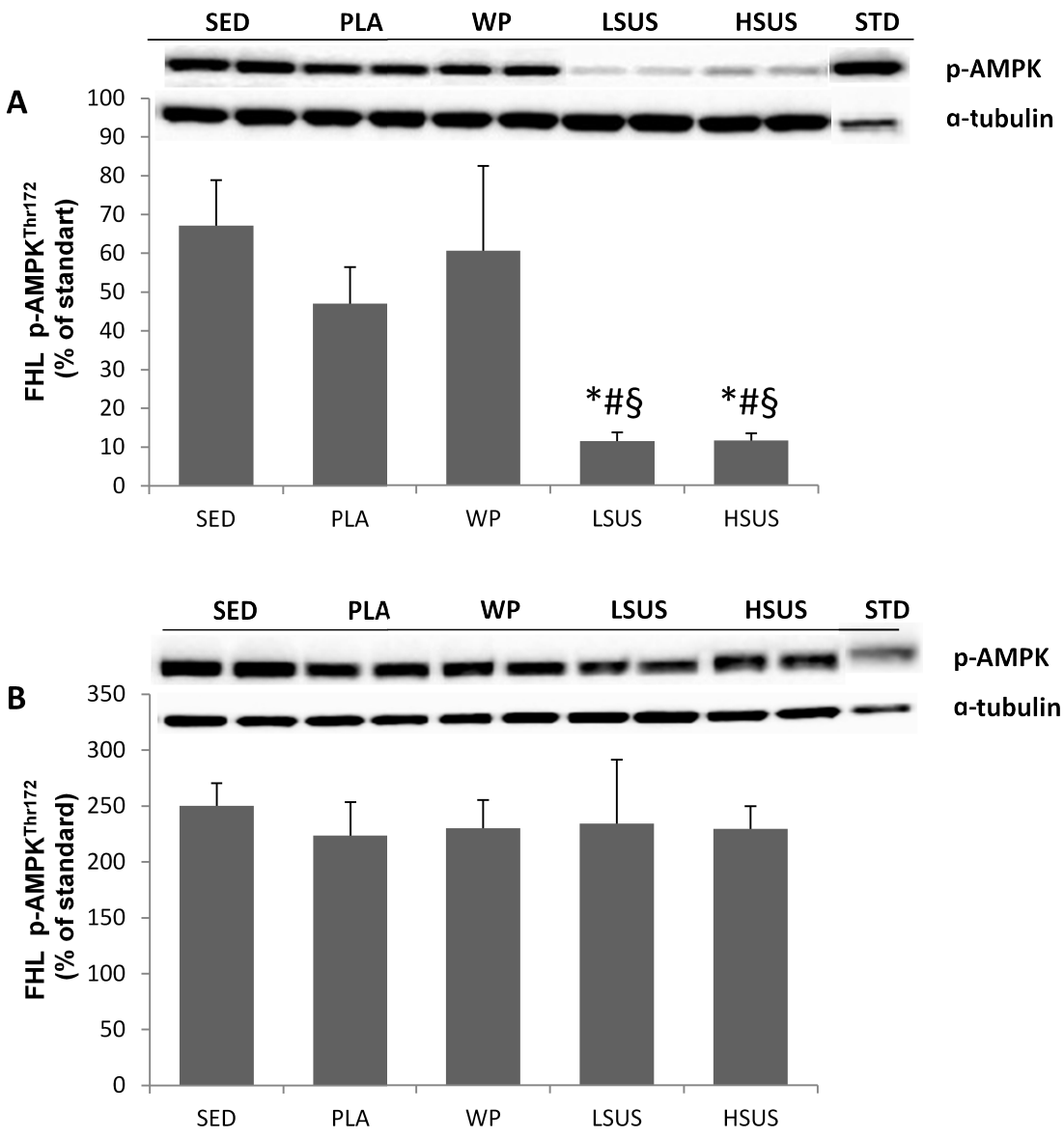

Figure 3.2. AMPK phosphorylation at Thr^{172}expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED. #, $p<0.05$ vs. PLA. §, $p<0.05$ vs. WP.

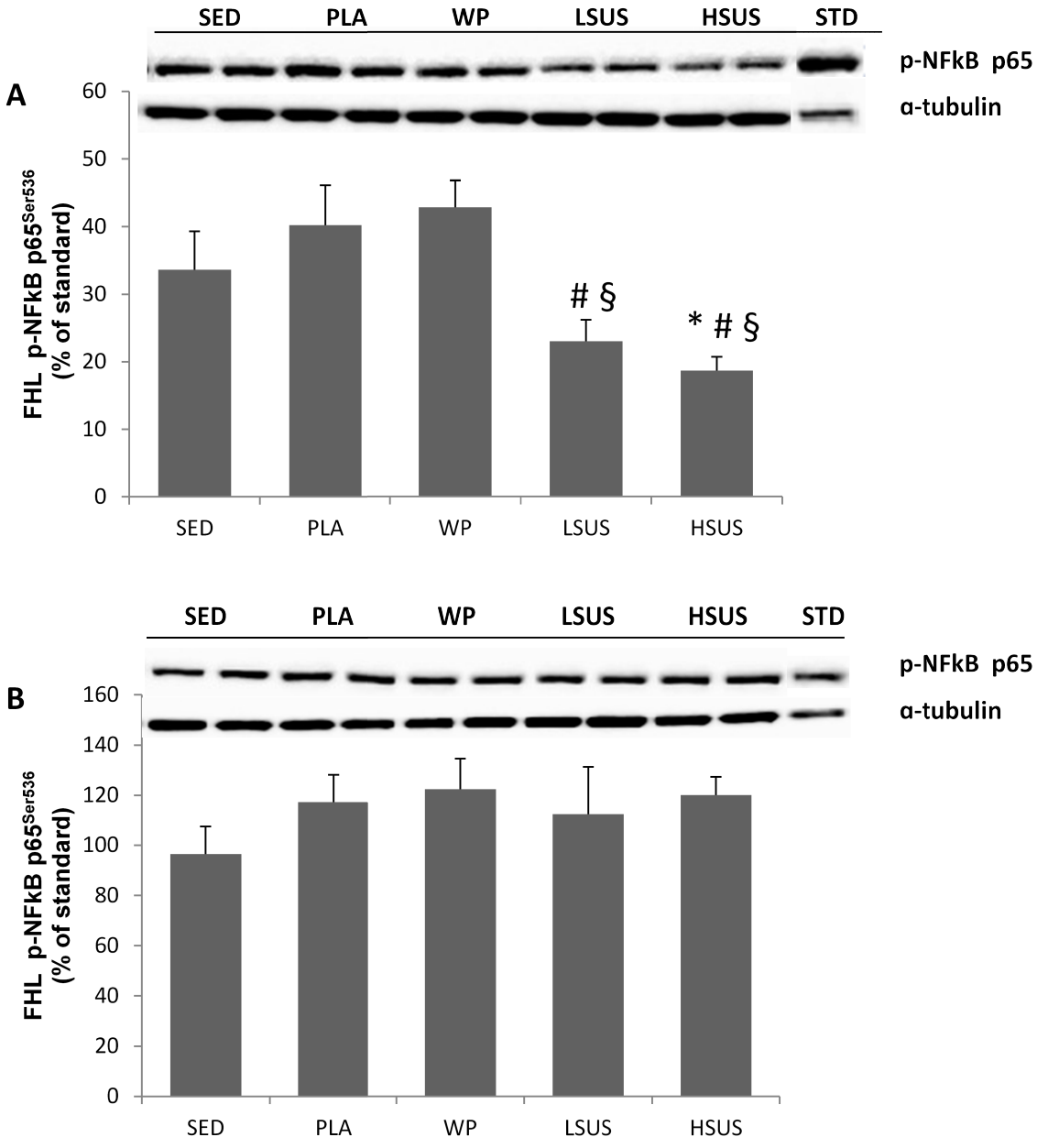

Figure 3.3. NF-kB p65 phosphorylation at Ser^{536} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED. #, $p<0.05$ vs. PLA. §, $p<0.05$ vs. WP.

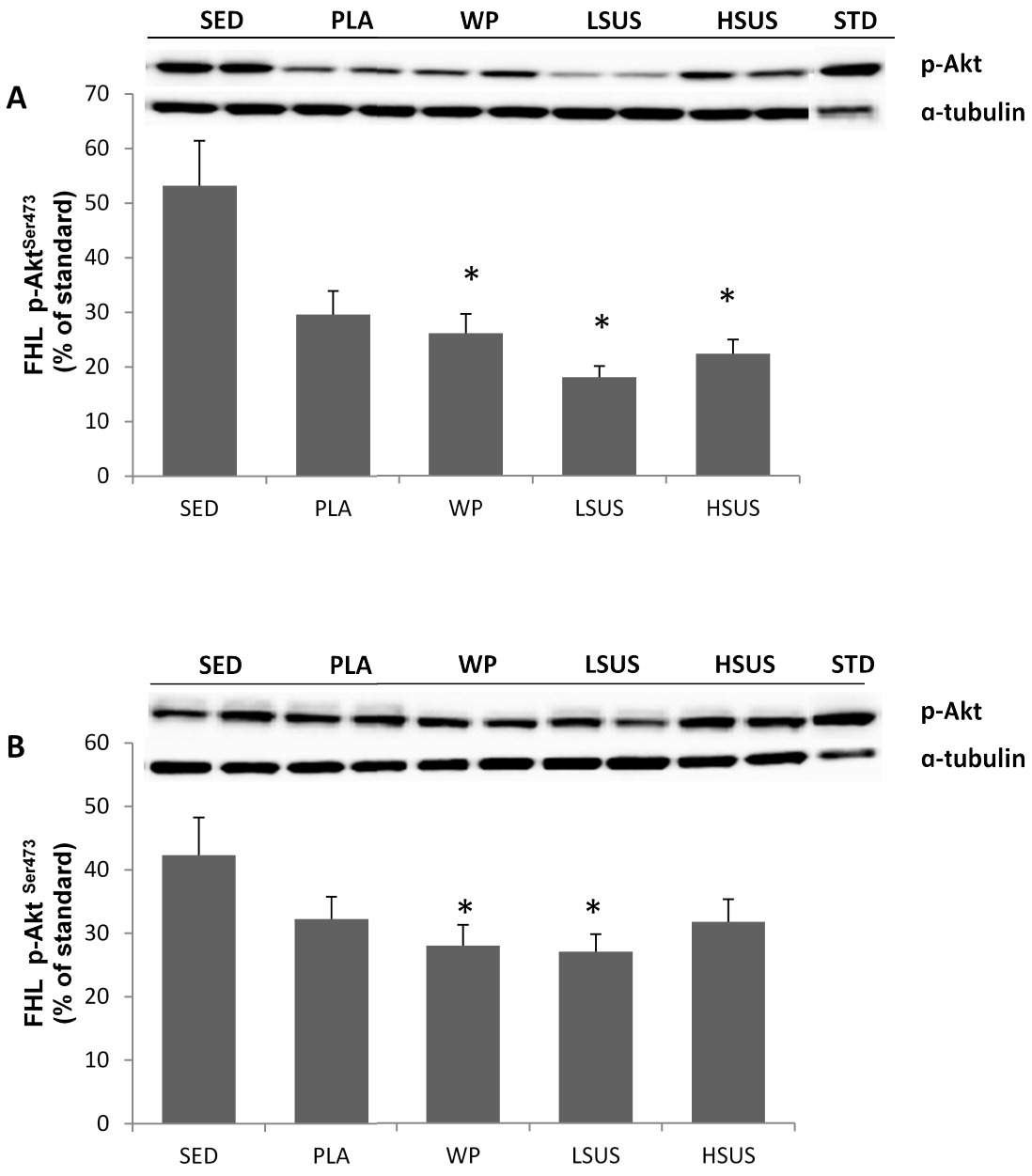

Figure 3.4. Akt phosphorylation at Ser^{473} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED.

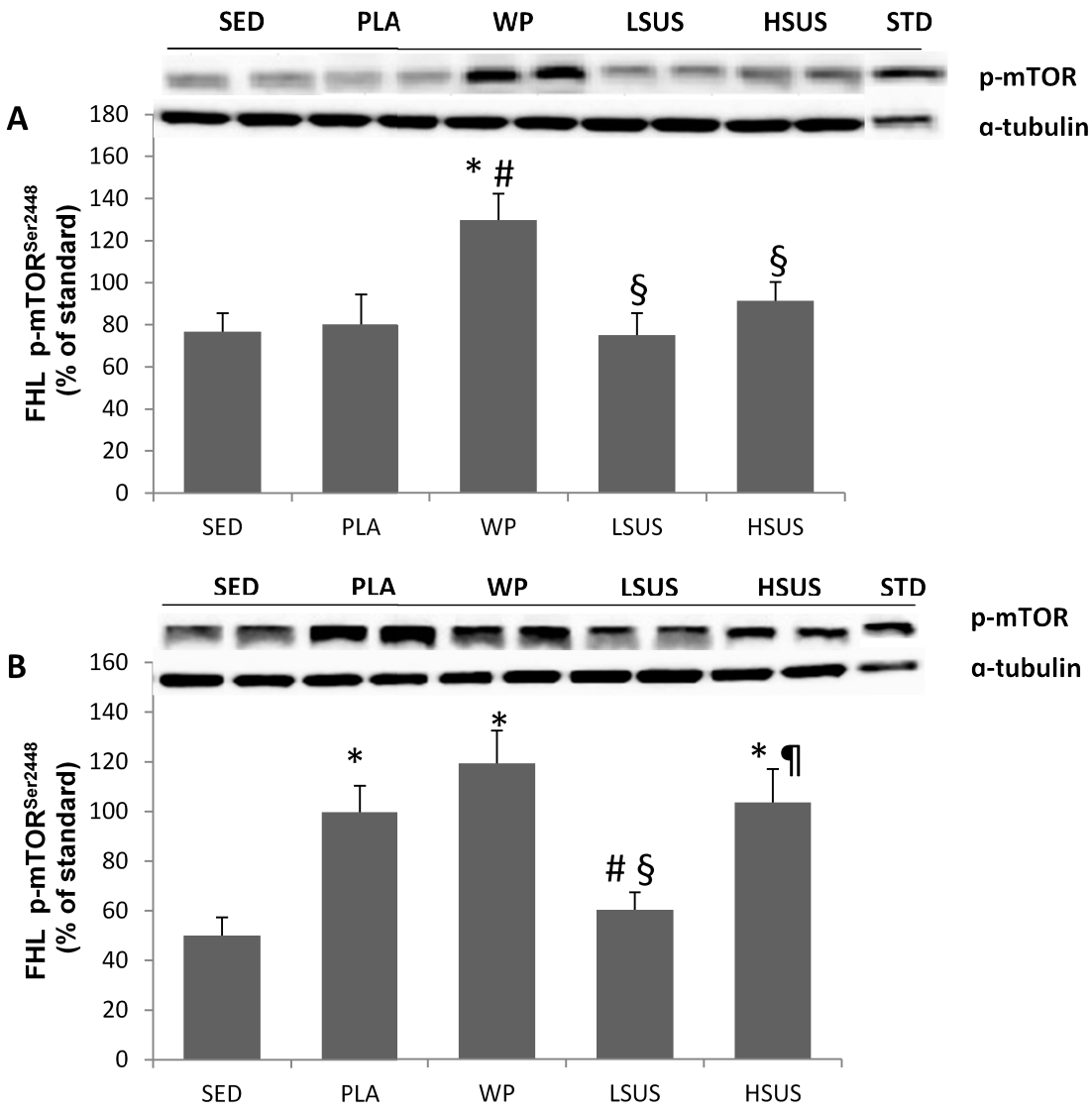

Figure 3.5. mTOR phosphorylation at Ser^{2448} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED. #, $p<0.05$ vs. PLA. §, $p<0.05$ vs. WP. ¶, $p<0.05$ vs. LSUS.

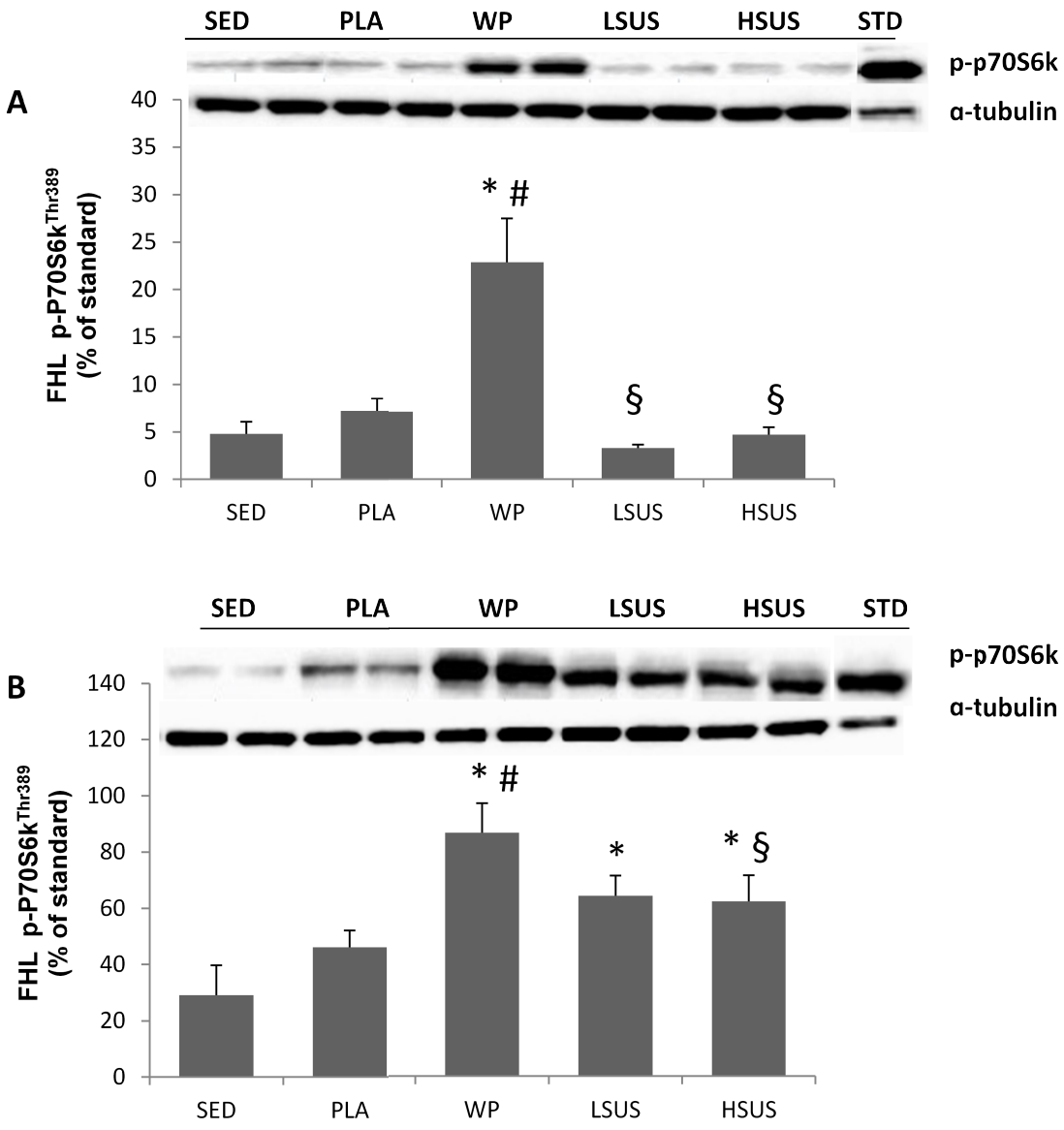

Figure 3.6. p70S6k phosphorylation at Thr^{389} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED. #, $p<0.05$ vs. PLA. §, $p<0.05$ vs. WP.

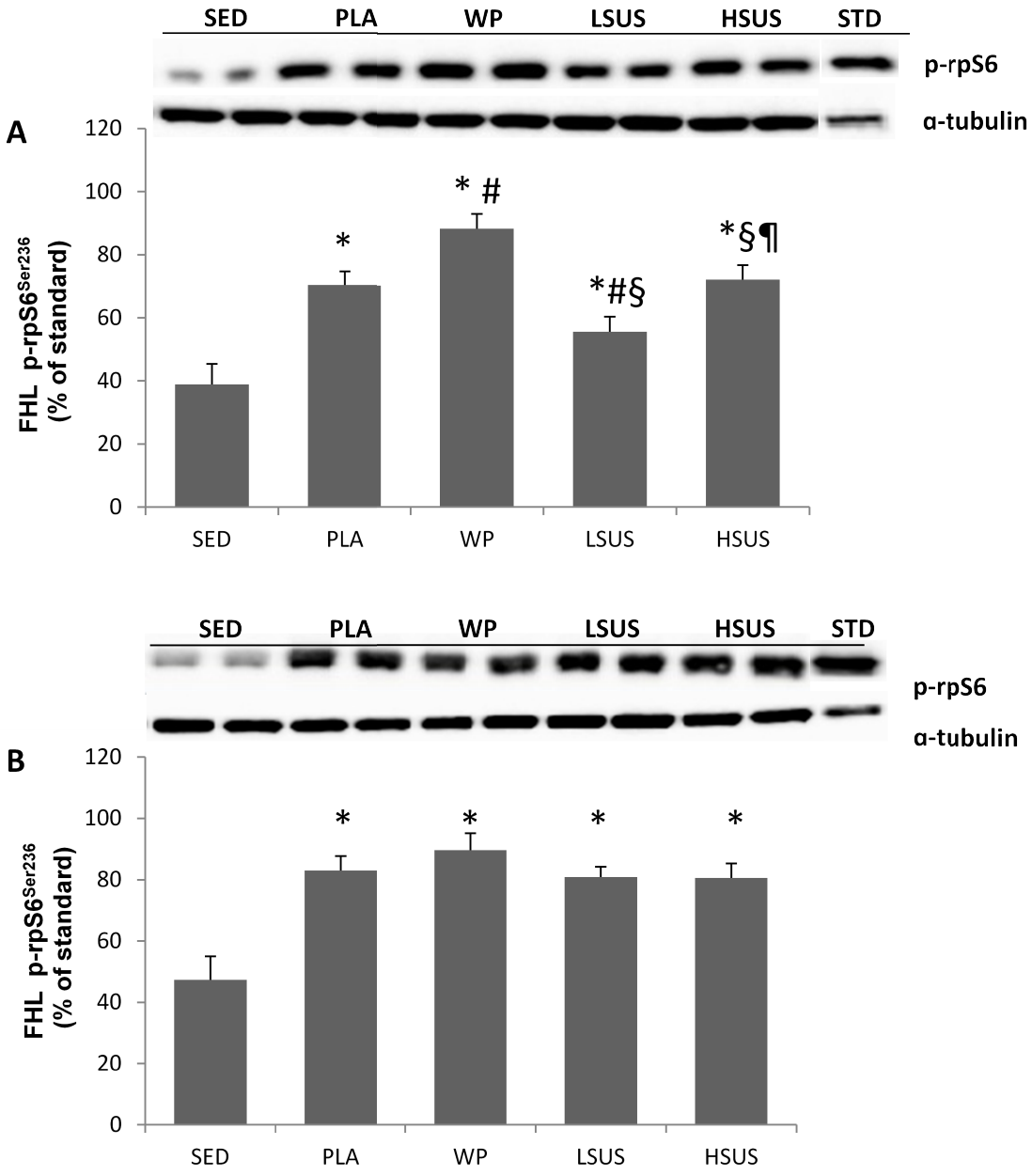

Figure 3.7. rpS6 phosphorylation at Ser^{236} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (A) and 40 min (B) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM (n=6-9 per group). *, $p<0.05$ vs. SED. #, $p<0.05$ vs. PLA. §, $p<0.05$ vs. WP. ¶, $p<0.05$ vs. LSUS.

Chapter IV: Co-ingestion of Carbohydrate and Whey Protein Accelerates Muscle Protein Synthesis during Early Recovery of Resistance Exercise

ABSTRACT

The objective of the study was to investigate whether co-ingestion of carbohydrate and protein as compared with protein alone augments muscle protein synthesis (MPS) during early exercise recovery. Two months old rats performed 10 repetitions of ladder climbing with 75% of body weight attached to their tails. Placebo (PLA), whey protein (WP), or whey protein plus carbohydrate (CP) were then given to rats by intubation. An additional group of sedentary rats (SED) was used as controls. Blood samples were collected immediately and at either 1 or 2 h after exercise. The flexor hallucis longus muscle was excised at 1 or 2 h post exercise for analysis of MPS and related signaling proteins. MPS was significantly increased by CP compared with PLA ($p<0.05$), and approached significance compared with WP at 1 h post exercise ($p=0.08$). CP yielded a greater phosphorylation of mTOR compared with SED at 1 h post exercise and SED and WP at 2 h post exercise. CP also increased phosphorylation of p70S6K compared with SED at 1 and 2 h post exercise. 4E-BP1 phosphorylation was inhibited by PLA at 1 h but elevated by WP and CP at 2 h post exercise relative to SED. The phosphorylation of AMPK was elevated by exercise at 1 h post exercise, and this elevated level was sustained only in the WP group at 2 h. The phosphorylation of Akt, GSK3, and eIF2Bε were unchanged by treatments. Plasma insulin was transiently increased by CP at 1 h post exercise. In conclusion, CP supplementation post exercise hastens MPS post exercise relative to PLA or WP by enhancing activation of the mTOR signaling pathway. This result may be related to a greater insulin response following CP supplementation.

INTRODUCTION

Resistance exercise (RE) is a potent stimulator of muscle protein synthesis (MPS), and the repeated activity can bring about skeletal muscle hypertrophy (45). However, some previous studies have suggested that MPS is inhibited or unchanged early after exercise (1, 24). When it comes to muscle protein breakdown (MPB), it was reported to be increased during RE and remain elevated for up to 24 h post exercise in the fasted state (5, 45). Under these conditions a negative muscle net protein balance can occur during the early recovery phase following RE (5). Therefore, nutritional supplementation immediately after exercise is crucial to increase MPS as well as inhibit MPB during the early phase of recovery. It is commonly accepted that protein supplementation provides sufficient amino acids and activation of the mammalian target of rapamycin (mTOR) signaling pathway to increase MPS after exercise (40, 54, 58). mTOR is a central kinase that integrates upstream signals from muscle contraction, amino acid (AA), and growth factors to stimulate MPS via mediating protein translation initiation. As such, a single bout of RE is able to activate the mTOR signaling pathway, which can be further potentiated by providing a post exercise protein supplement.

Interestingly, Anthony and colleagues reported that high intensity endurance exercise stymied muscle anabolism at early recovery of exercise, and protein supplementation could not recover the synthetic rate back to basal level (1). It is still unclear whether the same situation occurs after RE. Additionally, 20 g of high-quality protein supplementation has been reported to sufficiently maximize MPS post RE in young adults (40), while excessive protein consumption leads to increased AA oxidation. Hence, it may be of benefit to investigate new nutritional strategies to enhance MPS during the early phase of exercise recovery aside from supplementing post exercise with protein alone.

Recent studies suggest that adding carbohydrate (CHO) to a protein supplement may have potential benefits on muscle recovery after RE (26, 39, 41). Immediately after high intensity RE, the human body is under a catabolic state due to the reduction of insulin and the elevation of catabolic hormones, including cortisol and epinephrine. Insulin is a strong anabolic hormone. Numerous studies have clearly shown a suppressive effect of insulin on MPB (6, 13, 48) through inhibition of forkhead box 3A (FOXO3A) (19). Insulin also activates the mTOR

signaling pathway via activation of phosphatidylinositol 3-kinase (PI3k). However, the role of carbohydrate supplementation, a major insulin secretagogue, on MPS remains controversial. Some *in vitro* studies demonstrated that insulin significantly increased myotube protein synthesis (30, 32), but only a few *in vivo* studies have found a positive effect of insulin on MPS (21, 25). The ineffectiveness of insulin to stimulate MPS *in vivo*, may be due to a lack of AA availability as insulin increases AA clearance from the blood. However, co-ingestion of CHO and protein could possibly maximize muscle protein accretion post exercise by supplying a sufficient concentration of AA while also promoting a hyperinsulinemic state. To date, studies investigating the combined effects of CHO and protein/AA supplementation on MPS have reported inconsistent results (33, 39). Hence, the primary objective of this study was to investigate if adding CHO to a whey protein (WP) supplement results in a greater MPS during the early period of exercise recovery relative to WP alone. The present study also examined the phosphorylation state of signaling proteins in a manner that increase MPS and reduces MPB.

MATERIALS AND METHODS

Animals Eighty male Sprague-Dawley rats were obtained at two months of age from Charles River (Wilmington, MA). Rats were housed two per cage and provided standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21°C, with a reverse artificial 12:12 h dark-light cycle. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conform to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Exercise familiarization Following 1 week of acclimation, rats underwent 3 repeated sessions of ladder climbing to familiarize with the exercise protocol. Each session consisted of 4 trials and was separated by 1 day. The rats carried no weight during this familiarization period. The ladder was 1 meter height with an incline of 85° with 2 cm grid steps. After the initial familiarization, the rats then completed another 3 practice sessions of climbing separated by 1 day between each session with 50, 60 and 70% of their body mass attached to their tails,

respectively. The weight was secured to the tail with foam tape (3M Conan) and a Velcro strap. Rats were encouraged to climb by lightly tapping their tails with a bottle brush.

Experimental protocol Following an overnight fast, rats underwent an acute resistance exercise, as described in our previous study (55). Briefly, rats were placed on a climbing ladder to ascend 10 times with a weight equal to 75% of their body mass attached to the tail. There were two minutes rests between each climb. Upon the completion of the exercise protocol, each rat was wrapped separately in a towel. A 0.7 ml blood sample was obtained from the tips of their tails. Immediately after exercise, animals received either a placebo (PLA=ddH_2O), whey protein (WP=0.375 g/kg), or carbohydrate plus WP (CP=1.2 g/kg dextrose+0.375 g/kg WP) supplement by intubation. Twenty rats were used as sedentary controls (SED) and received an intubation of ddH_2O. All rats were randomly assigned to a treatment prior exercise familiarization. Rats in each treatment group were then subdivided by time of euthanasia, which occurred at 1 or 2 h post intubation (n=10). After intubation, rats were returned to their respective cage. Either 30 or 90 min after intubation, 0.04 μmol/g body weight of puromycin dissolved in PBS (pH=7.4) was given to the rats by intraperitoneal injection. Approximately, 20 min after the puromycin injection, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg of body weight). A second blood sample was collected, followed by the excision of the flexor hallucis longus (FHL) muscle at 1 or 2 h post intubation. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight).

Blood Analysis All blood samples were centrifuged at 3,000 g for 10 min at 4ºC with a FS-20 microtube rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). After centrifugation, the cleared plasma samples were stored at -80°C for later analysis of glucose, insulin, corticosterone, growth hormone (GH) and insulin-like growth factor (IGF)-1. Plasma glucose was determined using a colorimetric method, which employs glucose oxidase and a modified Trinder color reaction (no. 315, Sigma Chemical, St. Louis, MO). Plasma insulin was measured using a radioimmunoassay kit (Millipore Corporation, Billerica, MA) with CV<10%. The concentration of corticosterone was determined by an enzyme-linked immunosorbant assay kit (ELISA) with CV<10% (Enzo life sciences Inc. Ann Arbor, MI. Cat

ADI-900-097). Plasma GH (Millipore Corporation, Billerica, MA) and IGF-1 (Immunodiagnostic Systems Inc., Scottsdale, AZ) were measured using ELISA kits with CV<10% and read at dual wavelengths of 450nm and 630nm..

Western blot analysis Immunoblot analysis was performed as previously described (55). In brief, ~50 mg of muscle was homogenized in ice-cold homogenization buffer (20 mM HEPES, 2 mM EGTA, 50 mM sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DL-dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 mM sodiumorthovandate (Na_2VO_4)) at a 1:8 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). The homogenates were centrifuged at 14,000 g for 10 minutes at 4°C, and the supernatants were taken for measurement of total protein according to Lowry (36), and protein synthesis and the phosphorylation of designated cell signaling proteins by western blot. All supernatants were stored at -80 ºC until analyzed.

For western blots, supernatant samples containing 60µg protein were combined with an equal amount of Laemmli sample buffer (125mM Tris, 20% glycerol, 20% SDS, 0.25% bromophenol blue, and β-mercaptoethanol, pH 6.8) and boiled at 95ºC for 15 min in order to denature the muscle proteins (35). Then, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10-15% resolving gel at 130 V for 90 min or 90 V for 2.5 h (Bio-Rad Laboratories, Hercules, CA.). The resolved proteins were then electrically transferred onto a nitrocellulose membrane (pore size: 0.45 µm; GE Healthcare Life Sciences, Pittsburgh, PA.) using a wet transfer unit (Bio-Rad Laboratories, Hercules, CA.) at 90 V for 90 min. Ponceau S. (0.1% in 0.5% acetic acid) was used to verify the completeness of the transfer. The membranes were then washed in Tris-buffered saline (TBS) with 0.06% Tween20 (TTBS) to remove the Ponceau S. staining, and then the membranes were blocked in 7% nonfat milk in TTBS (blocking buffer) for 1 h at room temperature (RT). The membranes were then incubated with the appropriate primary antibody overnight at 4ºC. The targeted phosphorylated proteins were mTOR (Ser2448), 70 kDa ribosomal protein S6 kinase (p70S6k) (Thr389),

ribosomal protein S6 (rpS6) (Ser235/236), 4E binding protein 1 (4E-BP1) (γ isoform), protein kinase B (Akt) (Ser473), glycogen synthase kinase (GSK)-3α/β (ser21/9), eukaryotic initiation factor 2 (eIF2)-Bε (ser539), forkhead box (FOXO) 3A (Ser318/321), and 5' adenosine monophosphate-activated protein kinase (AMPK) (Thr172). Alpha-tubulin (α-tubulin) was used as an internal loading control. Anti-puromycin antibody was used to detect muscle protein synthesis according to a previous research (23). Antibodies for anti-p-eIF2Bε and anti-puromycin were purchased from EMD Millipore Corporation (EMD Millipore Corporation, Chicago, IL). All other antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). Following overnight primary antibody probing, all membranes were washed 5 min, 3 times with TTBS. The membrane incubated with anti-puromycin antibody was incubated with HRP-conjugated secondary anti-mouse IgG (EMD Millipore Corporation, Chicago, IL). All others were incubated with HRP-conjugated secondary anti-rabbit IgG (Cell Signaling Technology, Beverly, MA). After 3 additional 5 min washes, the membranes were visualized by enhanced chemi-luminescence (ECL) in accordance to the manufacture's instructions (Perkin Elmer, Boston, MA). All membranes were stripped and re-probed for α-tubulin as an internal loading control. All western blots were performed in duplicate for each muscle sample to ensure reproducibility (CV<8%). Images were then captured using a charge-coupled device camera in a ChemiDoc system (Bio-Rad, Hercules, CA). Intensity of each band was quantified with Quantity One analysis software (Bio-Rad) and expressed as a percentage of a standard.

Free puromycin concentration assay Free puromycin as a precursor can be delivered to the muscle and incorporated into nascent peptide chains. The measurement of free puromycin was used to normalize the value of MPS obtained from western blot analysis in the same sample. The analysis was conducted as described previously (23) with slight modifications. Approximately, 30 mg of muscle were homogenized in ice-cold homogenization buffer at a 1:15 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). A 250µl aliquot sample homogenate was precipitated with 28µl of 100% trichloroacetic acid and incubated for 30 min on ice followed by 5 min of centrifuge at 4,200g. This was followed by the addition of 15µl of Tris

buffer containing 1M Tris, 3M NaCl, and 1% Tween 20 at pH 7.0 and 30µl of 5.25M NaOH to 250µl supernatant to adjust the pH to ~9.0 (8.97-9.03). Next, the samples were filtered through a >3kDa filter (Amicon Ultra-0.5ml; Millipore, Carrigtwohill, Ireland) at 14,000g for 60 min. Meanwhile, a range of standards (0-40 pmol/100µl) were made and adjusted to pH 9.0. A 100µl sample or standard was added to a 96-well amine-binding maleic anhydride activated plate (Pierce; Thermo Fisher Scientific) in duplicate and rocked overnight at 4ºC. The next day, the plate was washed 4 times using PBS with 1% Tween 20 (PBST with pH7.0) and blocked with 1% BSA-PBST for 45 min at room temperature. 100µl of anti-puromycin antibody (clone 12D10, 1:38,400) was added to each well and incubate for 105 min at RT. Followed this incubation period, the wells were washed 3 times. Next, horseradish peroxidase-conjugated anti-mouse IgG Fc 2a (1:10,000; Millipore) was added to each well and incubated for 45min at RT. After another 4 washes, Ultra 3,3',5,5'-tetramethylbenzidine (TMB, Thermo Fisher) was added to each well and rocked for 15 min. The reaction was then stopped by 0.16 M sulfuric acid (Thermo Fisher). The absorbance was measured on a plate reader at a wavelength of 450nm. The concentration of free puromycin was calculated from a standard curve.

Statistical analysis Data obtained from western blot were analyzed as a percentage change relative to an insulin-stimulated rat tissue standard or anti-puromycin-injected rat tissue standard. A two-way analysis of variance (ANOVA) (time x treatment) was performed on a between-within mixed model design for the measurement in data obtained from plasma assays and data obtained from western blots. When a significant F test was identified, differences among means were determined using LSD post hoc analysis. Differences with $p < 0.05$ were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics v19.0 software (IBM Corporation, Armonk), and all data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Muscle protein synthesis

The relative value of puromycin labeled peptides from western blot analysis represented muscle protein synthesis (Figure 4.1.A). Free puromycin is a precursor delivered to the muscle

and incorporated into nascent peptide chains. There was no significant difference in levels of free puromycin among groups (Figure 4.1.C). In order to eliminate the possibility that changes in MPS were due to the differences in precursor uptake, protein synthesis was normalized to the concentration of free puromycin in the muscle (Figure 4.1.D). The result showed that exercise (PLA) transiently decreased MPS at 1 h post exercise compared to the sedentary (SED) ($p=0.08$). However, this inhibition on MPS was completely rescued by the CP treatment (Figure 4.1.D). CP also showed a higher trend of MPS than that of in the WP group at 1 h post exercise ($p=0.08$) (Figure 4.1.D). No significant difference was observed among treatments at 2 h post exercise (Figure 4.1.D).

Signaling pathways related MPS and MPB

The phosphorylation of mTOR was significantly increased by CP at 1 and 2 h post exercise compared with SED ($p<0.05$). mTOR phosphorylation was also higher in CP than PLA at 1 h, and higher than WP at 2 h (Figure 4.2.A). p70S6k phosphorylation was significantly elevated by CP at 1 h post exercise. At 2 h, p70S6k phosphorylation was further elevated by CP, but it did not differ from that in the PLA or WP (Figure 4.2.B). rpS6 is a downstream factor of p70S6k. Exercise significantly enhanced the phosphorylation of rpS6 both at 1 and 2 h post exercise ($p<0.05$). There was no difference in levels of rpS6 phosphorylation among all three exercise groups (Figure 4.2.C). The phosphorylation of 4E-BP1 was reduced by PLA at 1 h post exercise ($p<0.05$). WP and CP reversed this reduction completely at 1 h and further increased the phosphorylation of 4E-BP1 at 2 h compared with SED ($p<0.05$). The phosphorylation of 4E-BP1 also returned to the basal level at 2 h in the PLA group (Figure 4.2.D).

Akt is an upstream substrate of mTOR, but the phosphorylation of Akt did not correlate with the mTOR activation. Akt phosphorylation did not differ statistically across treatments at 1 or 2 h post exercise ($p>0.05$) (Figure 4.3.A). GSK3 is a downstream substrate of Akt. Neither GSK3 α nor β showed any significant differences among groups (Figure 4.3.B, C). Similarly, no significant difference in eIF2Bε phosphorylation was observed among groups at either 1 or 2 h post exercise (Figure 4.3.D).

The phosphorylation of AMPK and FOXO3A was significantly increased in all three exercise groups at 1 h post exercise (Figure 4.4.A, B). The phosphorylation of AMPK remained elevated at 2 h in WP ($p<0.05$) (Figure 4.4.A). The phosphorylation of FOXO3A was significantly increased in the CP compared with that in SED at 2 h post exercise ($p<0.05$) (Figure 4.4.B).

Glucose level and hormonal changes in plasma

There was no significant difference in plasma glucose among groups immediately post exercise ($p>0.05$) (Table 4.1.). At 1 h post exercise plasma glucose was higher in the CP than that in the WP group. At 2 h post exercise plasma glucose was significantly reduced in both PLA and WP compared with immediately after exercise, and these levels were lower than that in CP ($p<0.05$) (Table 4.1.). There was no difference in plasma insulin levels among groups immediately post exercise ($p>0.05$). However, plasma insulin was transiently elevated in the CP group at 1 h post exercise ($p<0.05$) (Table 4.1.). Plasma GH was significantly reduced immediately post exercise, but it was not different among groups at either 1 or 2 h post exercise (Table 4.1.). Plasma IGF-1 did not differ across treatments at any time point (Table 4.1.). Plasma corticosterone was significantly elevated by exercise ($p<0.05$), but returned to the basal level at 1 and 2 h post exercise (Table 4.1.).

DISCUSSION

It is commonly accepted that RE can induce muscle protein accretion primarily by stimulating MPS, and that this activation can remain elevated for many hours after a single bout of RE (14, 21, 45). However, within a short period of exercise recovery, MPS may be dampened in the fasted state even though the mTOR signaling pathway is activated (1, 24, 45). When the interval between exercise sessions is very short, an effective muscle recovery process can be of significant benefit. The major finding of this study is that co-ingestion of CHO plus WP rescued the reduction of MPS by exercise during the early recovery phase, and was associated with the activation of the mTOR signaling pathway.

At 1 h post exercise, MPS decreased if no supplement was provided. This is likely mediated through the elevated activation of AMPK and inactivation of 4E-BP1. Researchers suggested that AMPK activation might contribute to the inhibition of MPS during exercise and to the delayed activation of MPS during early post exercise recovery (16, 34). In the present study, phosphorylation of AMPK and inhibition of 4E-BP1 were significantly increased in PLA at 1 h post exercise relative to the SED group. However, the differences between these two groups disappeared at 2 h post exercise. The changes in these signaling proteins displayed a pattern similar to the changes in MPS. When the supplements were provided immediately after exercise, WP did not recover MPS at 1 h post exercise. Similar result was found by Anthony et al. when whey and soy protein were provided in an endurance exercise model (1). However, it is worth noting that CP supplementation significantly augmented MPS compared with that of PLA at 1 h post exercise. Moreover, MPS by CP showed a higher tendency than that in the SED and WP groups at 1 h post exercise. An early research paper compared independent and combined effects of AA and CHO over 3 h post exercise, and demonstrated that the coordinated effect of AA and CHO on MPS was roughly equal to the sum of their independent effects (39). Previous studies from our laboratory investigating the impact of protein and CHO on signaling proteins that control MPS and MPB post exercise also found that CP, compared with CHO or protein intake individually, had a greater effect on the activation of anabolic signaling proteins (26, 41).

Some investigators, however, were unable to find any beneficial effect of adding CHO to a post exercise protein supplementation on MPS. For example, Koopman and colleagues compared casein plus different doses of CHO (0, 0.15, 0.6 g/kg) on whole body protein synthesis and breakdown 6 h after RE (33). They did not observe any differences in either protein synthesis or breakdown treatments. Similarly, Staples et al. reported neither MPS nor MPB could be further affected by CHO plus WP relative to WP supplement alone 3 h of recovery from RE (51). The discrepancy among studies might be partly due to the types of protein intake, timing of protein synthesis measurement, dosages of supplementation, and animal model. In the present study, we did not observe any difference in MPS among PLA, WP, and CP groups at 2 h post exercise. Perhaps, the effect of CP supplement on MPS is very rapid and occurs only during the first hour of recovery unless additional supplementation is supplied at a later period.

Regarding to the underlying mechanisms for the changes in MPS, mTOR is considered an essential kinase in the regulation of MPS. Once mTOR is activated, it further phosphorylates two downstream factors, p70S6k and 4E-BP1 (20). p70S6k can further activate rpS6, which then increases capacity of protein synthesis. The present study showed that CP provided immediately post exercise yielded a greater increase in the phosphorylation of mTOR and p70S6k relative to the SED group at 1 h. The phosphorylation of mTOR was also higher in the CP compared with PLA at 1 h and approached being significantly higher than WP. At 2 h post exercise, phosphorylation of mTOR for CP was significantly greater than SED and WP. Phosphorylation of p70S6K in CP was significantly increased above SED at 1 and 2 h post exercise. PLA and WP did not increase the phosphorylation of p70S6k until 2 h post exercise. Similar results have been reported in both animal and human subjects (15, 41). For example, Dreyer et al. explored the effect of essential AA (EAA) with CHO consumption on MPS in relation to the mTOR signaling pathway after RE (15). Subjects were provided either a placebo or CHO+EAA supplement post exercise. Their results indicated that the EAA+CHO stimulated a greater MPS 2 h post exercise than placebo and this MPS was related to a greater phosphorylation of mTOR and p70S6k.

In the present study we found that exercise alone led to phosphorylation of rpS6 at both 1 and 2 h post exercise, which was not further affected by nutritional supplementation. This result was not surprising because other studies haves provided strong evidence that muscle contraction is able to activate rpS6 directly via 90-kDa ribosomal S6 kinase ($p90^{RSK}$) and without affecting p70S6k (10, 44, 47, 55). On the other hand, 4E-BP1 mediates binding of mRNA to the 40S ribosomal subunit (20). Hyperphosphorylation of 4E-BP1 dissociates itself from eIF-4E so that eIF-4E is able to bind to eIF4G and eIF4A to start the eIF4F complex (43, 56). This complex then becomes a bridge linking mRNA to the 43S pre-initiation complex, which increases rates of mRNA translation (1). As mentioned previously, phosphorylation of 4E-BP1 at 1 h post exercise was significantly restrained when no supplement was provided, but phosphorylation returned to the basal level at 2 h post exercise. When the supplements were provided, however, both WP and CP triggered a higher phosphorylation of 4E-BP1 relative to PLA at 1 h post exercise, and at 2 h post exercise phosphorylation levels were even higher than SED. The results suggest that WP

and CP supplementations were capable of inhibiting 4E-BP1 during the first hour of recovery and to maintain this inhibition for several hours.

Akt is an upstream substrate of mTOR. Akt can be phosphorylated via the insulin-dependent signaling pathway by phosphoinositide 3 (PI3)-kinase. The present study found that CP transiently increased plasma insulin levels at 1 h, whereas phosphorylation of Akt was not affected by any treatment. This suggests that the activation of mTOR by CP was not regulated through the insulin-signaling pathway. However, the phosphorylation and activation of Akt can be rapid and very transit. Therefore, there is the possibility that by 1 h post exercise it was too late to observe the phosphorylation of this protein. Glycogen synthase kinase (GSK) 3 is involved in another step of translation initiation. The activation of GSK3 phosphorylates eukaryotic translation initiation factor 2(eIF)-Bε to reduce its activity (29). Both phosphorylated Akt and p70S6k are capable of inhibiting GSK3α/β, leading to activation of eIF2Bε (29, 31). eIF2α then exchanges GDP for GTP on eIF2, which binds met-tRNA to the 40S ribosomal subunit and form the 43S pre-initiation complex (9). We, however, did not observe any changes in the phosphorylation levels of GSK3β, GSK3α, or eIF2Bε across the various treatments or times. These results are in agreement with other previous research (10, 57). Bolster et al. also did not observe changes in eIF2Bε activity or the phosphorylation of eIF2α during 1 h of recovery from RE (10). Wilkinson and colleagues reported that GSK3β remained unchanged 4 h after exercise in the fed state, but, eIF4E was activated (57). In contrast, Glover et al. found a substantial change in eIF2Bε dephosphorylation 6 h after exercise in a human study (22). Earlier animal studies also revealed that MPS was enhanced as late as 16 h after RE due to an elevation of eIF2Bε activity (17), but not the dissociation of eIF4E from 4E-BP1 (18). It is possible that the regulation of MPS by the GSK3-eIF2Bε dependent signaling pathway only occurs during the later stages of exercise recovery. Accordingly, our results suggest that during the early phase of recovery, the activation of mTOR and inhibition of 4E-BP1, regulated by nutritional supplementation, play a more important role in the stimulation of MPS than the GSK3-eIF2Bε signaling pathway.

AMPK is another upstream regulator of mTOR. In the present study, the phosphorylation of AMPK was significantly elevated at 1 h post exercise. This observation is supported by previous studies demonstrating that exercise alone can increase the phosphorylation of AMPK, and reduce MPS via inhibiting mTOR and decreasing 4E-BP1 phosphorylation (8, 16). Thus, the increased AMPK phosphorylation at 1 h post exercise may count for the lack of change in the mTOR phosphorylation and the dampened MPS in the PLA and WP groups as compared to SED. In contrast, the mTOR phosphorylation and MPS for CP were significantly increased at 1 h post exercise although AMPK phosphorylation was also increased. It is speculated that the elevation in plasma insulin following the CP supplement overrode the inhibitor effects of AMPK on mTOR via the insulin-signaling pathway. A weakness in this hypothesis is that CP supplementation had no effect on the phosphorylation of Akt. However, as pointed out earlier, the phosphorylation of Akt is quite transient and may have occurred prior to muscle removal. A second possibility is related to insulin-stimulated AA membrane transport. An increase in plasma insulin can increase muscle blood flow and muscle AA transport, and block muscle AA output (4, 46). Hence, early enhancement of MPS by CP in the present investigation could have resulted from a rapid increase of AA uptake, particularly L-leucine uptake, which in turn could have activated mTOR. Further research is required to confirm either hypothesis.

Interestingly, AMPK phosphorylation remained elevated at 2 h post exercise only following WP. Similarly, Iwanaka and colleague found that L-leucine increased contraction-induced glucose transport and the activity of AMPK (27). Why an increase in AMPK phosphorylation was not observed at 2 h post exercise following CP supplementation is not known.

Other growth factors were measured in our study. Plasma GH was dampened immediately post exercise, but no differences were observed at 1 and 2 h post exercise among all groups. Neither exercise nor nutrient supplementation affected IGF-1 plasma levels. Therefore, CP induced MPS was unlikely controlled by these hormones.

With regards to MPB, the ubiquitin-proteasome system is primarily responsible for protein degradation (28). During the entire process of protein degradation, E3 enzyme mediated

ubiquitin ligation is considered the rate-limiting step (7, 52). There are two crucial ubiquitin E3 ligases in the skeletal muscle, muscle atrophy F-box (MAFbx or atrogin1) and muscle ring-finger protein 1 (MuRF1). These ligases have been shown to stimulate muscle proteolysis (2). The activation of both E3 ligases is under the regulation of transcription factors such as FOXO3A in the nucleus (60). Once phosphorylated, FOXO3A is exported into the cytoplasm and becomes inactive. In the present study, the phosphorylation of FOXO3A was significantly elevated in all three exercise groups at 1 h post exercise, but only the CP group showed sustained phosphorylation of FOXO3A compared to the SED group at 2 h post exercise. Akt and AMPK mediate the phosphorylation of FOXO3A, but with opposing control over MPB (12, 42). In our results, Akt remained unchanged across time and treatment, and phosphorylation of AMPK did not follow the same pattern as FOXO3A.

In terms of the hormonal regulation on MPB, RE can place the body into a catabolic state by elevating cortisol and suppressing insulin secretion (53, 59). Insulin has been shown to suppress MPB (6, 13, 48) due to its inhibition on FOXO3A (19). A high level of cortisol can result in rapid muscle atrophy (49), possibly by dampening MPS (37, 50) and increasing MPB (38). In the present study, plasma corticosterone was elevated immediately after exercise, and then returned to the basal level 1 h post exercise. Similar findings have been reported in a human study from our laboratory in which it was found plasma cortisol did not differ within 6 h post exercise with or without CP supplementation (3). However, it was found that 24 h post exercise plasma cortical in the PLA group was significantly elevated above that found in the CP group (3). Therefore, our results, together with previous research, suggest that corticosterone may not affect MPS or MPB within the first 2 h of recovery. Although plasma insulin was increased by CP at 1 h post exercise, all exercise groups were found to have similar levels of phosphorylation of FOXO3A at 1 h post exercise and were not significantly different at 2 h post exercise. These results suggest that CP supplementation was unlikely contributing to the inhibition of MPB during the early hours of exercise recovery. It is possible, however, that CP supplementation could have a delayed inhibitory effect on MPB. For example, Borsheimet al. demonstrated that CHO induced a rapid increases in plasma glucose and insulin, but the effect of insulin on MPB was detected 3 h after supplementation, but not at 1 or 2 h after (11). In addition, our laboratory

previously reported that a difference in plasma cortisol could not be observed between PLA and CP groups until 24 h post exercise, which further supports a delayed response of CP on MPB (3).

In summary, adding CHO to a protein supplement accelerates MPS during the early period of exercise recovery compared with PLA and WP. This early enhancement in MPS could have been caused by a rise in plasma insulin, which in turn could have overridden the inhibitory effect of AMPK on the mTOR signaling pathway. Additional time course studies are warranted to determine changes in both MPS and MPB during extended recovery times. Although the exact mechanism is not known, our findings indicate that CP is more efficacious post resistance-exercise than protein alone. The results suggest that over time regular supplementation with CP post resistance-exercise would produce a greater training adaptation than PLA or WP.

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Table 4.1. Plasma glucose, insulin, GH, IGF-1, and corticosterone concentration at 0, 1, and 2 h post exercise

	Treatment			
	SED	PLA	WP	CP
Glucose (mM)				
0 h	5.37±0.19	5.78±0.19	5.73±0.20	5.88±0.22
1 h	5.67±0.13	5.63±0.27	5.11±0.22	6.05±0.25 §
2 h	5.11±0.27	4.71±0.27*f*	4.22±0.25* *f* ‡	5.69±0.29 †§
Insulin (pM)				
0 h	218.45±19.01	199.53±11.97	274.84±23.47	265.59±53.29
1 h	289.47±27.40	225.99±30.86	244.79±32.30	423.01±55.34†§ *f*
2 h	266.43±33.46	255.51±39.97	280.76±26.16	320.64±38.30
GH (ng/ml)				
0 h	9.61±2.90	0.80±0.22*	1.46±0.53*	1.83±0.55*
1 h	6.54±2.65	3.83±1.21	9.44±7.68	9.77±5.67
2 h	3.89±0.73	7.50±2.54	7.13±3.88	2.24±0.85
IGF-1 (ng/ml)				
0 h	815.89±30.03	843.49±45.87	760.91±23.71	809.29±46.81
1 h	823.43±53.42	712.86±50.44	677.50±38.37	696.20±55.40
2 h	723.41±55.82	772.45±47.33	729.08±46.57	700.11±58.99
Corticosterone (ng/ml)				
0 h	144.84±14.63	270.94±16.84*	240.67±19.83*	227.61±25.77*
1 h	157.33±14.90	169.57±13.08 *f*	161.99±27.01 *f*	155.65±26.06 *f*
2 h	117.35±17.47	127.43±14.78 *f*	116.13±21.80 *f*	119.12±19.49 *f*

Data are presented as mean ± SEM (n=10 per group). *f*, $p<0.05$ vs. 0 h in the same treatment. ‡, $p<0.05$ vs. 1 h in the same treatment.*, $p<0.05$ vs. SED at the same time point. †, $p<0.05$ vs. PLA at the same time point. §, $p<0.05$ vs. WP at the same time point.

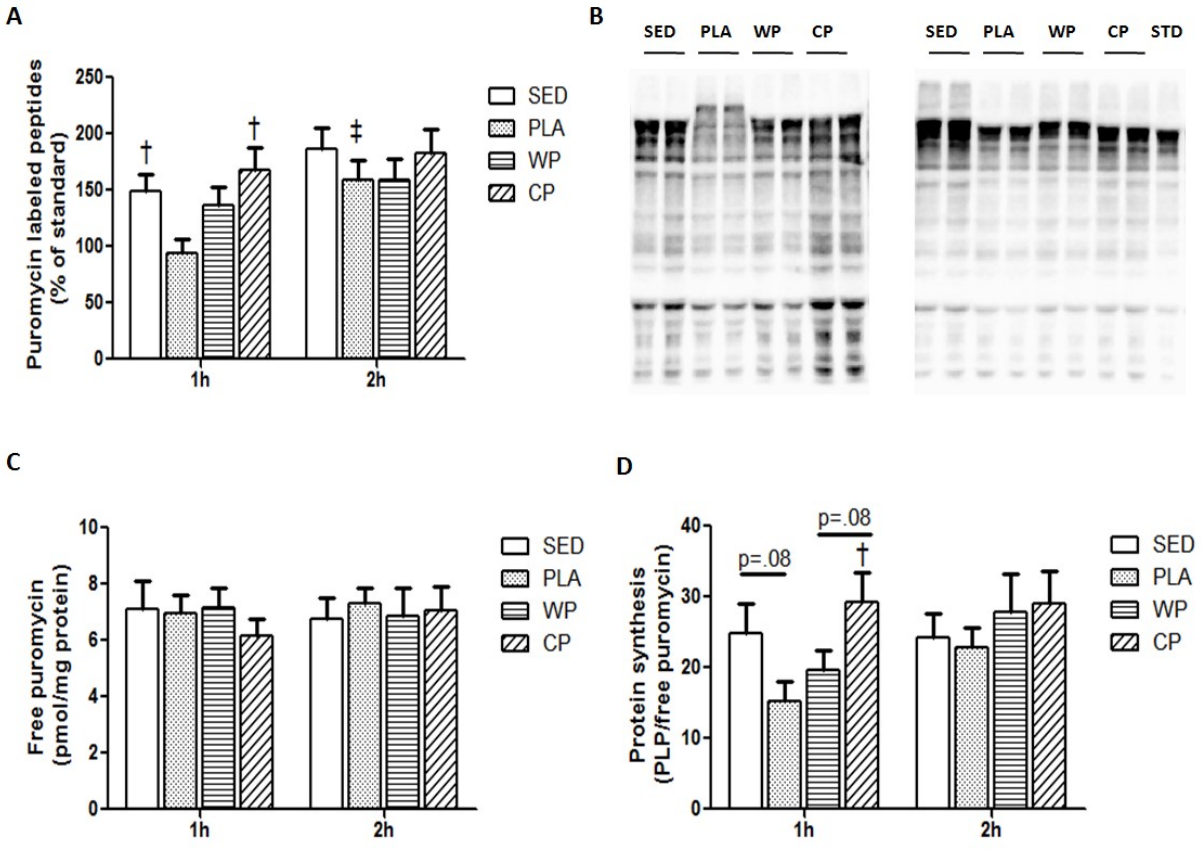

Figure 4.1. Muscle protein synthesis. A) Quantification of puromycin-labeled peptide, expressed as a percentage of a standard sample obtained from an exercise rat tissue. B) Representative image of western blot analysis for puromycin using a charge-coupled device camera. C) Free puromycin concentration measured as described. D) Muscle protein synthesis expressed using the value of puromycin labeled peptidesrelative to the free puromycin concentration in the same sample. All values are mean ± SEM (n=10 per group). †, $p<0.05$ vs. PLA. ‡, $p<0.05$ vs. 1 h.

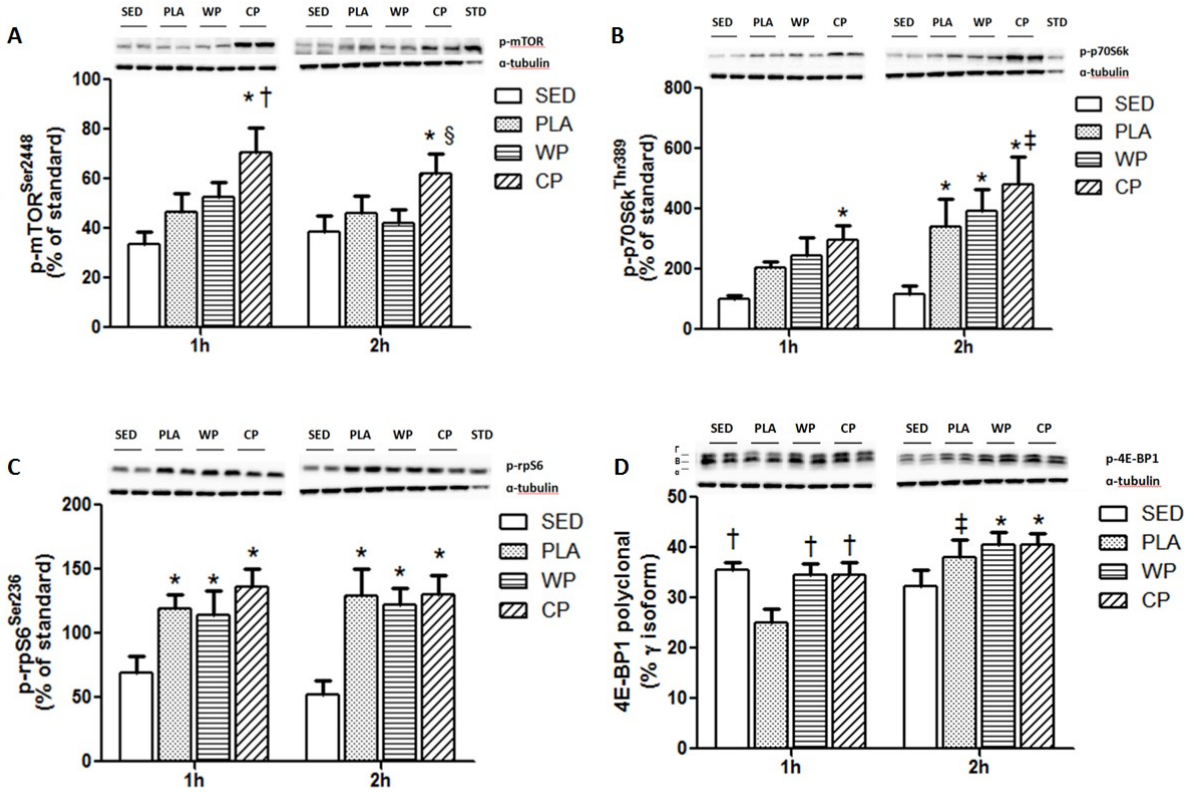

Figure 4.2. The phosphorylation of mTOR signaling pathways. A) mTOR phosphorylation at Ser^{2448} expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. B) p70S6k phosphorylation at Thr^{389}expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. C) rpS6 phosphorylation at Ser^{236} expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. D) 4E-BP1 phosphorylation expressed as a percentage of the gamma isoform. All values are mean ± SEM (n=10 per group).*, $p<0.05$ vs. SED. †, $p<0.05$ vs. PLA. §, $p<0.05$ vs. WP. ‡, $p<0.05$ vs. 1 h.

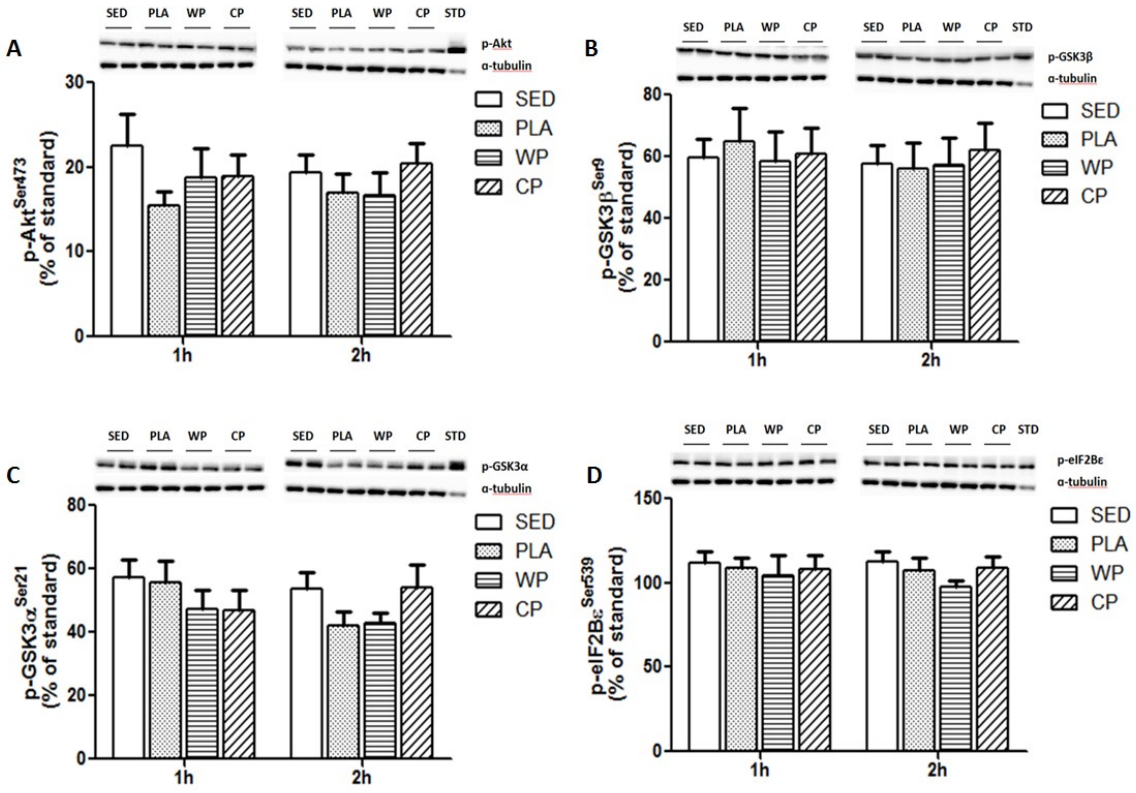

Figure 4.3. The phosphorylation of Akt-GSK signaling pathways. A) Akt phosphorylation at Ser^{473} expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. B) GSK3β phosphorylation at Ser^{9} expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. C) GSK3α phosphorylation at Ser^{21}expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. D) eIF2Bε phosphorylation at Ser^{539}expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. All values are mean ± SEM (n=10 per group).

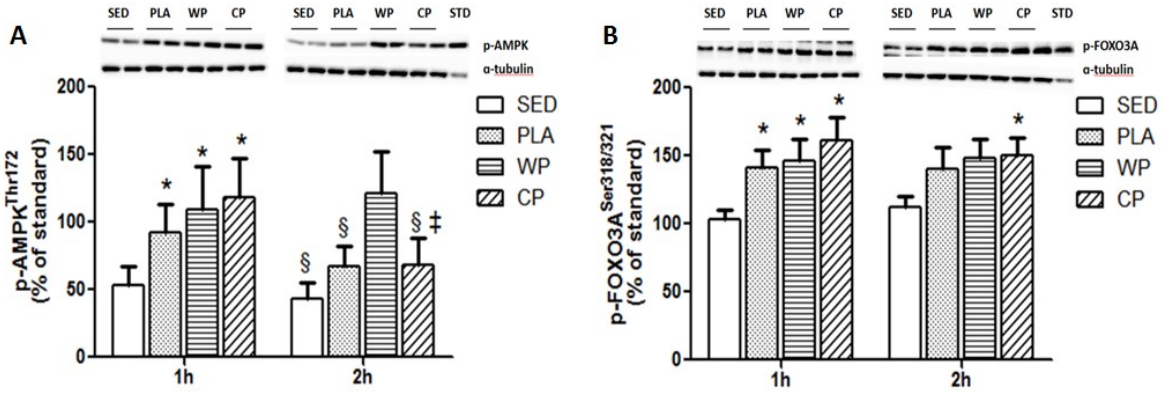

Figure 4.4. The phosphorylation of AMPK-FOXO3A signaling pathways. A) AMPK phosphorylation at Thr^{172} expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. B) FOXO3A phosphorylation at $Ser^{318/321}$ expressed as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. All values are mean ± SEM (n=10 per group).*, $p<0.05$ vs. SED. §, $p<0.05$ vs. WP. ‡, $p<0.05$ vs. 1 h.

Chapter V: Co-ingestion of Carbohydrate and Whey Protein Induces Greater Muscle Strength and Myofibrillar Protein Accumulation without a Requirement of Satellite Cell Activation

ABSTRACT

The present study compared the effects of a whey protein and carbohydrate (CP), whey protein (WP), and placebo (PLA) on resistance training adaptations. Two-month old rats were trained by ladder climbing every 3 days for 8 weeks. PLA, WP (WP = 0.5g/kg), or CP (Whey = 0.5g/kg, CHO= 1.2g/kg) was given immediately after each exercise session. Non-exercise rats were used as a sedentary control (SED). Total body composition was assessed before, middle, and end of training using dual-energy X-ray absorptiometry (DEXA). Blood samples were collected 22-24 h after the 1^{st}, 10^{th}, and 20^{th} exercise sessions. The flexor hallucis longus (FHL), a muscle used for climbing, was excised 24 h after the last exercise session and analyzed for muscle hypertrophy, total and myofibrillar protein, and proteins that regulate the activation of satellite cells (SC). Following training, maximal carrying capacity was significantly greater in CP than PLA and WP. This improved training performance was relevant to a greater increase in total and myofibrillar protein content in CP compared to PLA and WP groups. Resistance training significantly augmented FHL muscle mass and slightly reduced epididymis adipose mass, but total body composition did not differ across treatment groups. Muscle and fiber cross sectional area (CSA) were significantly increased by exercise training, with a concomitant increase in myonuclear domain, but there were no differences among exercise groups. CP significantly elevated IGF-1 expression over SED, but there were no significant differences in myostatin, Pax7, MyoD, and myogenin across treatment groups. There was also no difference in the number of total nuclei in each fiber CSA among groups. Corticosterone level was significantly elevated in PLA and WP over 8 weeks of training, whereas this change in corticosterone over time was not observed in the CP group. The results suggest that the greater improvement of maximal caring capacity for CP compared with PLA and WP was associated with a greater increase in myofibrillar protein content. The activation of SC did not appear to contribute to the observed gains in muscle hypertrophy and strength.

INTRODUCTION

Skeletal muscle is a highly plastic tissue in response to exercise, inactivity, changes in hormonal environment, and nutritional supplementation. An increase in muscle size and muscle strength is of paramount importance for health as well as physical performance. Resistance exercise training is the most effective strategy to induce muscle development, while evidence exists indicating that post exercise nutrient supplementation may augment muscle training adaptations (6, 14, 16, 41, 59).

Muscle development is controlled by the balance between muscle protein synthesis and protein degradation. The initial enlargement of muscle mass is primarily attributable to pre- and post-translational mechanisms, which stimulate muscle protein synthesis and inhibit protein degradation while correspondingly increasing the myonuclear domain (quantity of cytoplasm/number of nuclei within that cytoplasm) (46). According to the myonuclear domain theory (4), the area of cytoplasm that each nucleus can maintain is limited. Thus, expansion of the muscle cross sectional area (CSA) increases the demand of additional myonuclei once the myonuclear domain reaches a threshold level (46). Satellite cells (SC) have been identified as a major source of new myonuclei (50, 51). Myogenic regulatory factors (MRF), a superfamily of transcription factors, are composed of four members: MyoD, Myf5, myogenin, and MRF4. MyoD is predominantly upregulated during myogenic cell proliferation, and myogenin stimulates muscle differentiation (9, 54). These MRFs can be controlled by growth factors, such as insulin-like growth factor (IGF) -1 and myostatin. IGF-1 serves as a potential candidate for promoting skeletal muscle hypertrophy. It does not only activate the mammalian target of rapamycin (mTOR) signaling pathway contributing to protein synthesis, but also mediates the expression of MRFs to trigger SC proliferation and differentiation (5, 21, 46). Contrary to the actions of IGF-1, myostatin down-regulates muscle mass by inhibiting proliferation (60) and differentiation (33) of SC and reducing muscle growth. While the role of SC is commonly accepted during skeletal muscle regeneration, it is still under debate as to their relationship to muscle hypertrophy during resistance training (22, 25).

Another important strategy to enhance muscle hypertrophy and strength is nutritional supplementation. The ability of protein supplementation post exercise to enhance protein

synthesis is widely accepted (15, 39). Protein supplementation supplies the amino acids (AA) pool and regulates mRNA translation initiation via activating the mTOR signaling pathway (18, 40). During prolonged resistance training, protein supplementation has been found to increase the magnitude of muscle mass and strength development compared with the exercise stimulus alone (62). However, protein supplementation does not appear to attenuate protein degradation, which is also elevated during resistance exercise (11, 48). In addition, an increase in plasma cortisol during strenuous exercise may also counter an increase in muscle protein synthesis (32). Carbohydrate (CHO) supplementation has been shown to reduce the exercise-induce rise in cortisol (8, 59). Recently, we found that adding CHO to a post exercise protein supplementation accelerated muscle protein synthesis acutely [unpublished results]. Moreover, several resistance training studies suggest muscle mass and strength development may be enhanced when a CHO/protein supplement is provided around the time of each exercise session compared with either provided alone (10, 38). However, the few studies that have investigated the combined effects of CHO and protein supplementation on MPS have provided inconsistent results (10, 31, 38, 56). Therefore, the first aim of this study was to investigate whether adding carbohydrate to a post exercise protein supplement would induce a greater muscle hypertrophy and strength development than protein supplementation alone. The second aim of the study was to determine the influence of exercise and post exercise nutrition on SC activation, and their involvement in muscle development during resistance exercise training.

MATERIALS AND METHODS

Animals A total of 31 male Sprague-Dawley rats were obtained at approximately 2 months of age from Charles River (Wilmington, MA). Rats were singly housed in their cages in order to monitor diets and feeding patterns. PVC pipes were used as an environmental enrichment in each cage. Rats were provided standard laboratory chow (Prolab RMH 1000 5P07) and water ad libitum. The temperature of the animal room was maintained at 21 °C. A reverse artificial 12 h of dark-light cycle was set with the light phase from 8:00 pm to 8:00 am. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conform to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Exercise familiarization The resistance exercise training program employed consisted of ladder climbing. The ladder was 1 m high with 2 cm grid steps on an incline of 85°. Following one week of acclimation to their new environment, each rat underwent ladder climbing with three repeated sessions separated by one day between each session to familiarize them with the exercise protocol. During these three sessions, rats carried no weights. Rats also completed three practice sessions of climbing separated by one day between each session with 50, 60, and 70% of their body mass attached to their tails, respectively. The weight was attached at the base of the tail with foam tape (3 m Conan) and a Velcro strap. Rats were encouraged to climb by lightly tapping their tails with a bottlebrush.

Experimental design Following familiarization with the ladder climbing, rats began a high-intensity progressive resistance exercise regimen according to Hornberger and Farrar (28). In brief, the initial climb consisted of carrying a load equal to 75% of the rat's body weight. Upon successful completion of climbing the ladder with this load, an additional 30 g weight was added to the tail of the rat and the climb repeated after 2 min of rest. This procedure was repeated until a load was reached, which prevented the rat from completing the climb. In the subsequent training session, the first 4 ladder climbs consisted of 50%, 75%, 90%, and 100% of maximum load, respectively. Then, an additional 30 g load was progressively added until a new maximal carrying capacity (indicative of strength) was established. The training regimen was repeated every 3 days for 8 weeks, which consisted of a total of 20 exercise sessions. Whey hydrolysate (WP = 0.5 g/kg) (#8360, Hilmar Ingredients, Hilmar, CA), whey plus carbohydrate (CP: Whey =0.5 g/kg, CHO = 1.2 g/kg), or placebo (PLA = 8 ml/kg water) was provided by intubation immediately after each exercise regimen. Food was withdrawn 3 h before and after each exercise session with water provided ad libitum. Seven non-exercised rats were used as a sedentary group and received an intubation of water (8 ml/kg) during the times of training. The day before 1st exercise session, the days after the 9th and 19th exercise sessions, the rats were fasted for 3 hours and body mass obtained. Body composition was then determined via dual-energy X-ray absorptiometry (DEXA). Blood was collected 22-24 h after the 1st, 10th, and 20th exercise sessions. Following the last blood collection, rats were anesthetized to remove the flexor hallucis longus (FHL) from both legs and the epididymis adipose tissue. Muscle and adipose tissue

weights were obtained quickly. Then, the muscle from the left leg was embedded in optimal cutting temperature (OCT) medium and frozen in cooled isopentane for later determination of fiber cross sectional area (CSA), quantification of myonuclei, and myonuclear domain. The FHL from the right leg was freeze clamped in liquid nitrogen, and stored at -80°C for later determination of protein expression of IGF-1, myostatin, Pax 7, MyoD, and Myogenin, and total and myofibrillar protein content.

Body composition DEXA (enCORE 14.10, GE Healthcare, Madison, WI) was used to determine the body composition of each rat. On the day of testing, rats were fasted for 3 hours and then anesthetized via intraperitoneal injections of ketamine (70mg/kg) and xylazine (7mg/kg). Each rat was then placed in a supine position with attention to body alignment on a platform grid. The forelimbs and hindlimbs of the rat were placed perpendicular to the long axis of the body. The tail was positioned in a left curve toward the head so the entire body was contained within the scan area. Body mass, bone mineral density, fat mass, lean mass， and percent body composition were obtained.

Blood Analysis Approximately, 22-24 h after exercise sessions 1, 10, and 20, 0.7 ml of blood was collected into a 1.5 ml test tube containing 50 µl of EDTA (24 mg/ml, pH 7.4). All collected blood samples were centrifuged for 10 min at 3,000 g at 4ºC with a FS-20 microtube rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). After centrifugation, the plasma samples were placed in capped microfuge tubes and stored at -80°C for later analysis of corticosterone. The concentration of corticosterone was determined by an enzyme-linked immunosorbant assay kit (ELISA) with CV<10% (Enzo life sciences Inc. Ann Arbor, MI. Cat ADI-900-097).

Morphological analysis Frozen OCT embedded muscle was transversely cut into 10µm sections on a cryostat (Leica cm1900; Leica Microsystems Inc., Buffalo Grove, IL) set at -20°C. Sections were mounted on glass slide. Hematoxylin and eosin (H&E) staining (Fisher scientific Inc.) was performed to identify muscle fiber CSA and the number of myonuclei. Slides were observed under a light microscope (Nikon Diaphot, Nikon Corp.; Tokyo, Japan) with a 20x objective lens for fiber CSA and with a 40x objective lens for quantification of nuclei and myonuclear domain. Three random histological fields were collected in each muscle for analysis.

Images were then taken using a mounted digital camera, and measured and counted using Image J software.

Determination of muscle cross-sectional area The following formula was used to calculate muscle CSA.

Muscle CSA (mm^2) = muscle mass (mg) * cos θ (0.944) / [density (1.056mg/mm^3) * muscle length (mm) * (FL/ML)]

Immunoblot analysis Immunoblot analysis was performed as previously described (61). The frozen muscle samples weighing 80~100 mg were homogenized in ice-cold homogenization buffer (20 mM HEPES, 2 mM EGTA, 50 Mm sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DL-dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 mM sodium orthovandate (Na_2VO_4) at a 1:8 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). The crude muscle homogenates were centrifuged at 14,000 g for 10 minutes at 4°C, and the supernatants were taken for measurements of protein concentration. Aliquots of muscle homogenates were stored at -80 ºC until analyzed.

Muscle samples (60μg) were combined with an equal amount of Laemmli sample buffer (125 mM Tris, 20 % glycerol, 20 % SDS, 0.25 % bromophenol blue, and β-mercaptoethanol, pH 6.8) and boiled at 95°C for 10 min in order to denature muscle proteins. Samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10-15% resolving gel at 100 V for 75 min (Bio-Rad Laboratories, Hercules, CA.). The resolved proteins were then electrically transferred onto a nitrocellulose membrane (pore size: 0.45 μm; GE Healthcare Life Sciences, Pittsburgh, PA) using a wet transfer unit (Bio-Rad Laboratories, Hercules, CA) at 90 V for 60 min. The membranes were blocked in 7 % nonfat milk in Tris-buffered saline with 0.06 % Tween20 (TTBS) for 30 min at room temperature (RT). Then, the membranes were incubated with the appropriate primary antibody overnight at 4°C. The targeted proteins were IGF-1 (sc-9013), myostatin (sc-134345), pax 7 (sc-81975), myoD (sc-71629), and myogenin (sc-12732). The above antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc. Dallas, TX). Alpha-tubulin was used as an internal loading control (Cell Signaling Technology, Beverly, MA). Following overnight primary antibody

probing, all membranes were washed 5 min with TTBS three times. Then, the membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) or anti-mouse IgG (EMD Millipore Corporation, Chicago, IL). After another three washes using TTBS, the membranes were visualized by enhanced chemi-luminescence (ECL) in accordance with the manufacturer's instructions (Perkin Elmer, Boston, MA). All western blots were performed in duplicate for each muscle sample to ensure reproducibility (CV<10%). Images were then captured using a charge-coupled device camera in a ChemiDoc system (Bio-Rad, Hercules, CA). Intensity of each band was quantified with Quantity One analysis software (Bio-Rad) and expressed as a percentage of a standard.

Total and myofibrillar protein measurements Myofibrillar protein measurement was described previously (28). In brief, 50 mg muscle was homogenized in ice-cold solution containing 8.5% sucrose, 5 mM EGTA, 50 mM KCl, and 100 mM $MgCl_2$ using a Polytron homogenizer (Virtishear, Virtis, Gardiner, NY, USA). An aliquot of the crude muscle homogenate (0.1ml) was used to determine total protein concentration. The remaining homogenate was centrifuged at 2500 g for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in a solution (pH 6.8) containing 5 mM EGTA, 100 mM KCl, 5 mM $MgCl_2$, and 0.05% Triton X-100. The suspension was then centrifuged at 2500 g for 10 min at 4°C and the supernatant was discarded. This step was repeated two more times. The remaining pellet was washed in solution (pH 6.8) containing 5 mM EGTA and 100 mM KCl and centrifuged at 2500 g for 10 min at 4°C. This step was repeated one more time. The myofibrillar pellet was then resuspended in a solution (pH 7.4) containing 150 mM KCl and 5 mM tris-hydroxymethyl aminomethane. This suspension was used to determine myofibrillar protein concentration. Total and myofibrillar protein concentrations were determined by a modified Lowry Protein Assay (35).

Statistical approach A repeated analysis of variance (ANOVA) was performed on a between-within mixed model designs (treatment x time) for the measurements of body composition, maximal carrying capacity, and corticosterone. When the interactive effect (treatment x time) was statistically significant, differences among means were determined using Fisher's LSD post hoc analysis. A one-way ANOVA was used for all the other data, and Fisher's LSD post hoc test was performed to compare mean differences among treatments. Differences

with p-values ≤ 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics v19.0 software (IBM Corporation, Armonk), and all data was expressed as mean ± standard error of the mean (SEM).

RESULTS

Rat physical characteristics

Body mass, FHL muscle mass, epididymis adipose mass, and daily food and caloric consumptions are presented in table 5.1.. There were no differences in initial body mass, daily food and caloric consumptions across treatment groups. After 8 weeks of training, all rats had significant increases in body mass. The gains in body mass, for the exercise groups, however, were significantly lower than occurred in the sedentary (SED) group. Body mass gains did not differ among the exercise groups. Likewise, resistance training significantly augmented FHL muscle mass, but the muscle mass did not differ among exercise groups. Both placebo (PLA) ($p = 0.05$) and CP ($p = 0.06$) groups showed a trend for lower epididymis adipose mass relative to the SED group. In spite of mass differences in the FHL muscle and epididymis adipose tissue after 8 weeks of training, total body composition measured by DEXA did not differ among groups (Figure 5.1.). Percent of fat body mass was elevated whereas percent of lean body mass was reduced in all treatment groups after 4 weeks, and did not change during the subsequent 8 weeks of training (Figure 5.1.A, B). Additionally, there were no differences in percent fat or lean body mass across treatment groups (Figure 5.1.A, B). Changes in fat and lean mass showed a similar pattern as percent body composition (Figure 5.1.C, D). However, compared to week 4, SED and WP groups had a higher fat mass at week 8, and all groups had a greater increase in lean mass at week 8 (Figure 5.1.C, D).

Maximal carrying capacity

After 8 weeks of resistance training, rats in the PLA group increased their maximal carrying capacity by 2.7-fold (from 475±10 g to 1,280±37 g). Rats receiving WP immediately after each exercise session also increased their maximal carrying capacity by 2.74-fold (from 462 g± 13 to 1,260± 49 g). Rats receiving CP immediately after each exercise session increased their

maximal carrying capacity by 3.04-fold (from 461±14 g to 1,390±54 g) (Figure 5.2., 5.3.). The percentage increase in maximal carrying capacity was significantly greater in the CP group compared with the PLA and the WP groups (Figure 5.3.).

Muscle and fiber CSA

Eight weeks of resistance training produced a larger FHL and FHL muscle fiber cross sectional area (CSA) relative to the FHL of the SED group. Nevertheless, no significant differences were observed among the three exercise groups for either muscle mass or fiber CSA (Figure 5.4.A, B).

Total and myofibrillar proteins

To investigate the underlying mechanisms on increases in maximal carrying capacity, total and myofibrillar proteins were measured. Exercise training did not affect total protein concentration (Figure 5.5.A), but total protein content for CP was significantly increased above SED (Figure 5.5.B), with a trend for PLA to be also greater than SED ($p = 0.07$). No difference occurred among the exercise trained groups. From the total protein extracted for each muscle, we determined the myofibrillar protein component. Our results showed myofibrillar protein per g muscle from CP was significantly greater than SED (Figure 5.5.C). Myofibrillar protein of all 3 exercise groups was greater than SED when myofibrillar protein content was expressed as mg/muscle (Figure 5.5.D). Also, myofibillar protein content of CP was significantly greater than WP, and approached significance relative to PLA ($p = 0.06$).

Signaling proteins that regulate the activation of SC

Expression of proteins involved in the activation of satellite cells was then investigated using western blot. IGF-1 is a growth factor stimulating muscle protein synthesis and myogenesis. The results suggested that CP elicited a greater IGF-1 protein expression compared with SED (Figure 5.6.A). No other differences were noted for IGF-1 across treatments. Myostatin, another growth regulator, which plays a role in the down-regulation of muscle mass hypertrophy was investigated. However, no significant differences were found for myostatin

protein expression across treatment groups (Figure 5.6.B). To investigate the possibility of satellite cell activation with training, protein expression of Pax 7, Myo D and Myogenin were examined (Figure 5.6. C, D, E). Neither exercise training nor nutrient supplementation appeared to have an effect on these proteins. To eliminate the possibility that the activation of satellite cells was not seen due to a missed time point, number of nuclei per observed FHL muscle fiber was measured. However, the amount of nuclei per fiber did not differ across treatments (Figure 5.7.A), whereas nuclear domain per nucleus was significantly augmented by resistance training (Figure 5.7.B).

Corticosterone level

Plasma corticosterone level was measured 22-24 hours after the 1st (week 0), 10th (week 4), and 20th (week 8) exercise sessions. Corticosterone level was significantly elevated by exercise at week 8 compared to week 0 for the PLA and WP groups (Figure 5.8.). Corticosterone level also showed a significant increase from week 4 to week 8 in the PLA group. There was no difference in corticosterone level in the CP group over the 8 weeks of training, and there were no differences between exercise groups at any time point.

DISCUSSION

The primary finding of the present study was that maximum carrying capacity，indicative of muscle strength, was significantly greater in the CP group than the PLA or WP groups following 8 weeks of resistance training. Maximal carrying capacity during the first 4 weeks did not differ among exercise groups, and it may be explained by neural adaptation (57). The subsequent gains in carrying capacity were greater for CP than for the PLA and WP groups. CHO seemed to play a role in muscle protein accretion and strength. During the last week of training, however, the gain in the maximal carrying capacity became progressively more difficult. This phenomenon might be attributed to a “ceiling effect” where rat genetic potential is reached (45, 52).

Previous studies have clearly demonstrated that FHL muscle in lower extremity was highly responsive to this resistance training model (28, 34). In line with these literatures, our

study showed that FHL muscle mass was significantly increased by exercise training, but did not differ among exercise groups. Also, while FHL muscle mass was enhanced by exercise training, a single muscle hypertrophy was not sufficient to impact changes of total body composition. Muscle hypertrophy only occurred in the active muscle during the training. Therefore, no significant treatment differences were detected in the percent of fat and lean mass. Interestingly, percent of fat mass was increased after 4 weeks in all groups while percent of lean mass was correspondingly reduced. To our knowledge, no evidence exists to show this phenomenon in animal study. Two months old young adult healthy rats were used in our study. Over 8 weeks of training, rats were at puberty stage, which age range is comparable to 12-18 years old in human (7, 53). It has been well established that the puberty stage in human results in rapid body growth, increased weight, and fat deposition (49). Therefore, it was not surprising to observe an increased percent of fat mass in the present study. The absolute value of fat mass measured by DEXA in SED and WP was elevated from week 4 to week 8, which pattern was not seen in the PLA and the CP groups. Additionally, a slight, nonsignificant lower epididymis adipose mass was detected with PLA and CP relative to SED.

Resistance training significantly increased muscle and fiber CSA leading to an increase in FHL muscle mass, indicative of muscle hypertrophy. Muscle hypertrophy could be due to the accumulation of protein content in the muscle or edema-induced muscle swelling. In the present study, total protein concentration did not differ across groups. Total protein content for CP was significantly elevated above SED. PLA and WP groups also showed a slight increase in total protein content than SED. These data indicate that muscle hypertrophy is most likely due to the accumulation of protein content, but we cannot rule out the possibility of muscle edema, which remained determined in future studies. Muscle protein pools consist of myofibril, mitochondria, and sarcoplasm, etc. Among the different types of proteins, myofibrillar protein contains myosin and actin contractile proteins, and their accumulation can add to muscular strength. Our results demonstrated that myofibrillar protein content was enhanced for CP over the SED or the WP group. CP also showed a higher myofibrillar protein content trend relative to PLA ($p=.06$). Despite that such an increase in myofibrillar protein for CP did not result in a greater increase in

size of muscle mass compared to that occurred in PLA or WP, CP induced rise in myofibrllar protein content seemed to bring about a greater strength relative to the other two exercise groups.

The net balance between muscle protein synthesis and protein degradation determines the accumulation of myofibrillar protein and muscle size. The underlying mechanism of increasing myofibrillar protein was then investigated in the present study. Our result showed that muscle IGF-1 protein expression was upregulated in the CP group compared to the SED group. IGF-1 has been demonstrated to have autocrine/paracrine functions within muscle fibers (1, 24). It is capable of stimulating protein synthesis and satellite cell (SC) activation (23, 26). Although not investigated directly in this study, an acute study from our laboratory clearly demonstrated that CP accelerated MPS via activating mTOR signaling pathway compared to exercise alone. Moreover, Lee and colleagues found that protein synthesis was the source of muscle hypertrophy via increased IGF-1 expression in muscle (34). These results, along with the finding of the present study, support the hypothesis that CP can augment protein synthesis via increasing IGF-1 protein expression in skeletal muscle, as compared to the other two exercise groups.

From another point of view, elevated cortisol level post exercise has enormous implications for strength athletes. The harder the resistance training is, the greater the cortisol is released, and the greater protein degradation occurs (32). Thus, the generation of cortisol during high intensity of RE can lead to negative net protein balance and muscle damage (27). In the current study, we observed that exercise significantly elevated plasma corticosterone level at the end of week 8 over week 0 and week 4 in PLA, and corticosterone was also increased for WP in week 8 relative to week 0. As such, the elevation of circulating corticosterone may compensate for the anabolic effect caused by resistance training. In contrast, plasma corticosterone did not differ over 8 weeks of training in the CP group. This result is in agreement with the findings in human studies, which reported an attenuation of cortisol level by CHO intake after acute or chronic resistance exercise (8, 10). Therefore, our results reveal that CHO may attenuate MPB over 8 weeks of resistance training, and it can explain a higher myofibrillar protein concentration and content in the CP group. As mentioned previously, corticosterone is a strong catabolic hormone causing muscle damage, so elevated level of corticosterone in the PLA and the WP

groups at the end of 8 weeks of training may cause damaged sarcolemmas, and consequently lead to muscle edema to some extent. This could also account for our findings that CP resulted in a greater strength than the PLA and the WP groups but no significant differences existed in muscle CSA among training groups.

In addition to increased protein accumulation, muscle hypertrophy may also be accompanied by an increase in the number of myonuclei in response to muscle loading (3). As mentioned above, muscle IGF-1 contributes to the activation of SC. SC represents muscle precursor cells. Its activation, proliferation and differentiation provide new myonuclei in the muscle fiber. This crucial role of SC in muscle hypertrophy was first found in a study utilizing gamma-irradiation to kill SC (50). In this study, muscle size was not able to be increased by overload in the irradiated rats due to the lack of SC (50). A later research by Patrella et al. reported that a greater hypertrophy was driven by a greater SC activation (46). Some myogenic regulatory factors (MRF) are responsible for the activation of SC. The increased expression of IGF-1 has been reported to mediate MRFs expression and to enhance myoblast proliferation and differentiation (5, 21). On the other hand, myostatin, a member of the transforming growth factor-β superfamily, plays a role in the down-regulation of muscle mass change. In the current study, however, changes of Pax 7, MyoD, and myogenin were not observed among all groups. Early research provided strong evidence showing that 8 weeks of resistance training using weight lifting model induced an increased mRNA expression of MyoD and myogenin (2), but the protein expression was not examined. One possible reason that no differences in MRFs protein expression was observed may be due to a missed activating window, because these protein expressions were only examined at 24 h after last exercise session in this study. To further investigate whether SC has been fused into the myofiber for muscle hypertrophy, the number of nuclei per FHL muscle fiber CSA was measured. The amount of nuclei per fiber CSA did not differ across treatment groups, though the myonuclear domain was increased in the exercise groups. These results indicate that resistance training promotes protein synthesis without additional nuclei involvement. Although some studies have reported an increase in the amount of nuclei per muscle fiber by resistance training (42, 47), other studies failed to observe the same change (30, 36). This discrepancy may be due to the intensity of exercise training, the magnitude

of muscle hypertrophic response, muscle damage, and genetic factors. An early investigation examined muscle hypertrophy using surgical synergistic ablation in soleus muscle (55). The significant increased in the number of SC only occurred in the first stage when muscle fiber was severely disrupted and muscle regeneration was required. However, during the second stage of increased muscle activity, 30% increase in muscle hypertrophy was not accompanied by the activation of SC (55). The myonuclei fusion by SCs may be required to provide sufficient cytoplasmic space for muscle protein expansion only when the myonuclear domain reaches a ceiling size, below which the stimulation of MPS is probably the sole factor to induce hypertrophy (46). Lee and colleagues also pointed out that 8 weeks of resistance training was not sufficient to cause SC fusion to the existing fibers by detecting the lack of central nuclei in the training group (34). Therefore, the necessity of SCs relies on the magnitude of muscle hypertrophy. The lack of changes in the MRF and total number of observed nuclei in the current study suggest that our training load may not be adequate to induce the new myonuclei infusion by activating SC. In agreement with our finding, McCarthy et al. observed the same increases in muscle mass induced by mechanical overload between normal mice and SC-depleted mice (37). Myonuclear domain was expanded without an increase in myonuclei in hypertrophic muscle. Moreover, the ladder-climbing regimen used in our study was relatively modest in intensity compared to some other training protocols (20, 58), so muscle damage may not be severe to result in the SC activation.

It was widely reported that consumption of additional protein supplementation after exercise led to an augmentation of protein synthesis and subsequent muscle hypertrophy (19, 44). Compared to casein and other whey protein isolate, whey hydrolysate has been shown to hasten muscle recovery after exercise and achieved substantially greater gains in lean mass and muscle strength (13, 17). Surprisingly, WP provided immediately after each exercise session did not stimulate a greater increase in muscle maximal carrying capacity, muscle mass, and myofibrillar protein compared to resistance training alone in the present study. There are several possible explanations for the discrepancy found in our study and other literatures. First, a daily supply of AAs may be adequate to promote muscle hypertrophy and no additional protein supplementation was needed. Second, WP was provided after each exercise session rather than every day. Therefore, average daily protein intake between the PLA and WP groups did not

differ (4.54±0.05 g / rat in the PLA group versus 4.68±0.12g / rat in the WP group). Third, some other studies did not see any effect of protein supplementation on muscle adaptation (29, 43, 52), suggesting that the potential benefit of post-exercise protein supplementation may be affected by intensity of training, nutritional states, and timing (12, 44).

In summary, postexercise CP supplementation increased the magnitude of maximal carrying capacity following 8 weeks of resistance training. This greater increase in strength by CP, as compared to the PLA or the WP group, appears to be due to higher myofibrillar protein content in the FHL muscle. However, muscle hypertrophy in response to resistance training was not sufficient to involve additional nuclei infusion into muscle fibers. We conclude that providing CHO to a protein supplement can be recommended for the postexercise nutrition to promote greater muscle strength during prolonged resistance training.

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Table 5.1. Food consumption, total calories, body mass, FHL muscle mass, and epididymis adipose tissue mass

	SED	PLA	WP	CP
Daily food consumption (g)	29.0±0.4	27.7±0.3	27.6±0.7	27.2±0.8
Supplement			0.5g/kg	0.5+1.2 g/kg
Total calories (kcal/d)	102.4±1.4	97.9±1.0	97.7±2.6	97.2±3
Initial body weight (g)	376.7±14.2	394.9±9.6	394.3±10.4	373.5±7.1
Final body weight (g)	552.6±8.6 †	518.3±14.5 *†	529.7±16.9 *†	513.4±12.2 *†
Gains in body weight (g)	190.6±8.7	135.8±9.0 *	148.0±9.8 *	139.9±14.7 *
FHL (mg)	743.1±20.2	851.4±19.9 *	829.8±14.6 *	842.3±18.6 *
Epididymis adipose tissue (g)	9.6±0.6	7.7±0.93 (p=.05)	8.9±0.7	7.8±0.2 (p=.06)

Data are presented as mean ± SEM (n = 7 - 8 per group). †, $p \leq 0.05$ vs. initial body weight in the same treatment. *, $p \leq 0.05$ vs. SED at the same time point.

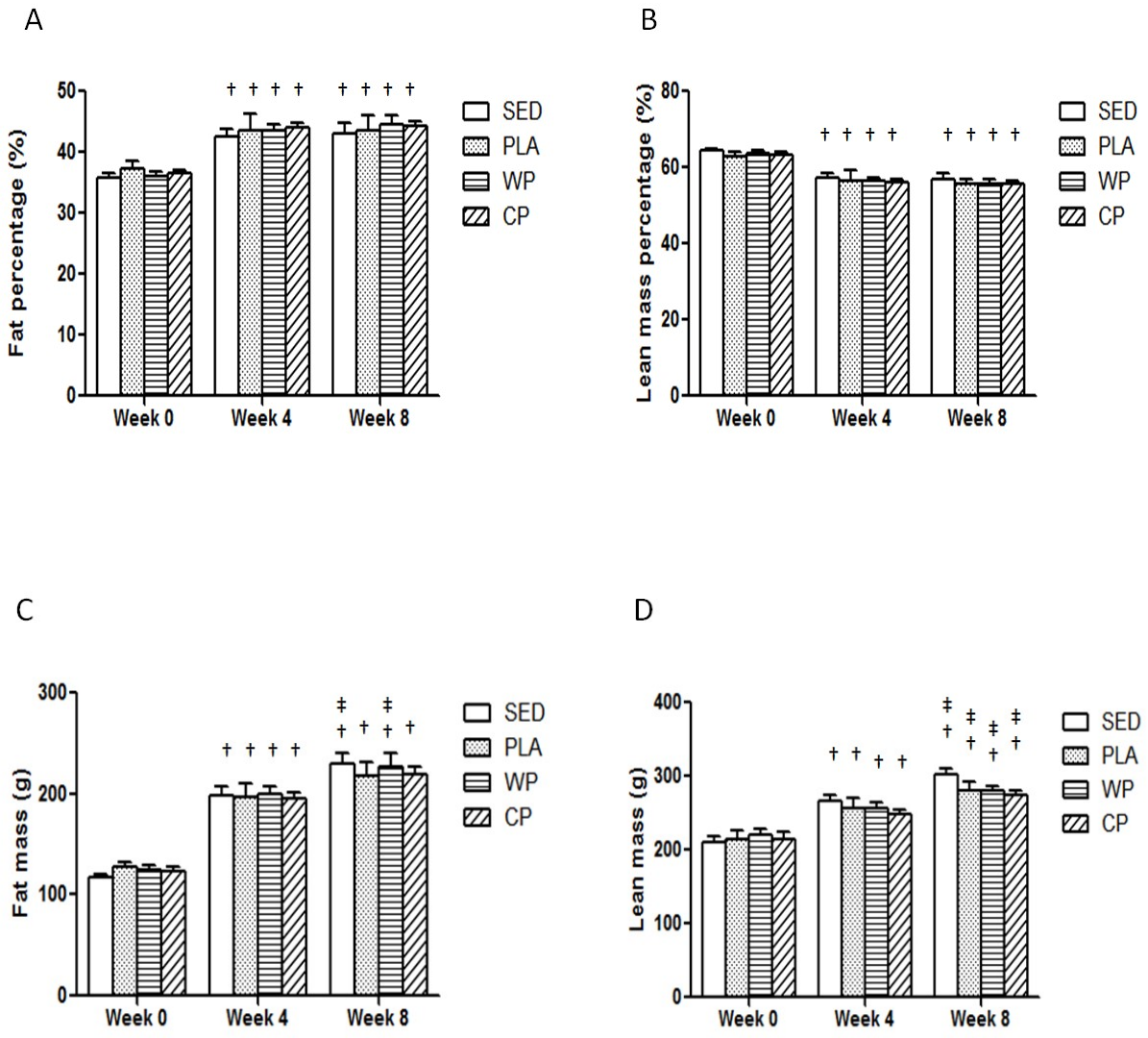

Figure 5.1. Effect of resistance training and nutrients on total body composition measured by DEXA. A) Fat percentage. B) Lean mass percentage. C) Fat mass. D) Lean mass. Data are presented as mean ± SEM (n= 7 - 8 per group). †, $p \leq 0.05$ vs. week 0 in the same treatment. ‡, $p \leq 0.05$ vs. week 4 in the same treatment.

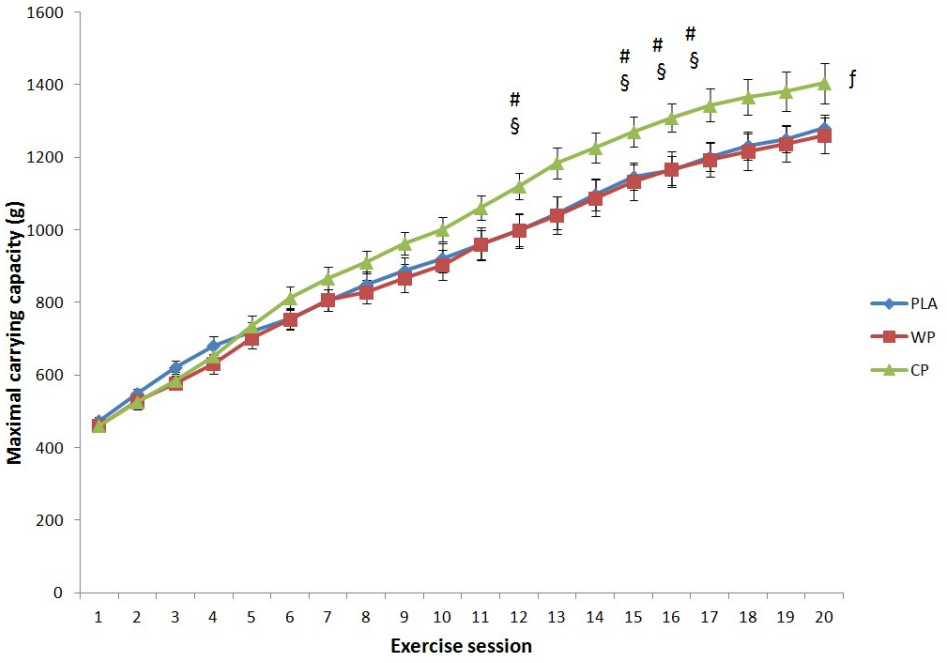

Figure 5.2. Maximal carrying load per training session over 8 weeks. Data are presented as mean ± SEM (n= 7 - 8 per group). *ƒ*, significant treatment effect in CP vs. PLA and WP. #, $p \leq 0.05$ vs. PLA. §, $p \leq 0.05$ vs. WP.

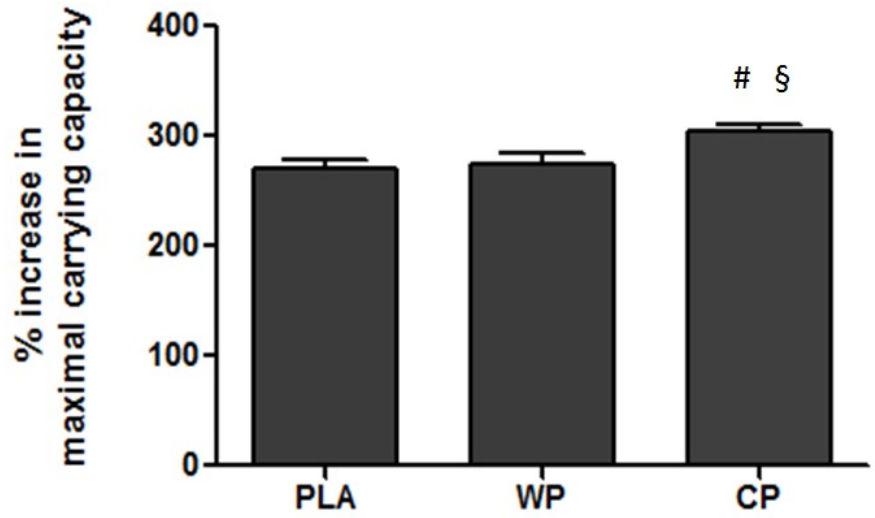

Figure 5.3. Percentage increase in maximal carrying capacity over 8 weeks of training. Data are presented as mean ± SEM (n = 7 - 8 per group). #, $p \leq 0.05$ vs. PLA. §, $p \leq 0.05$ vs. WP.

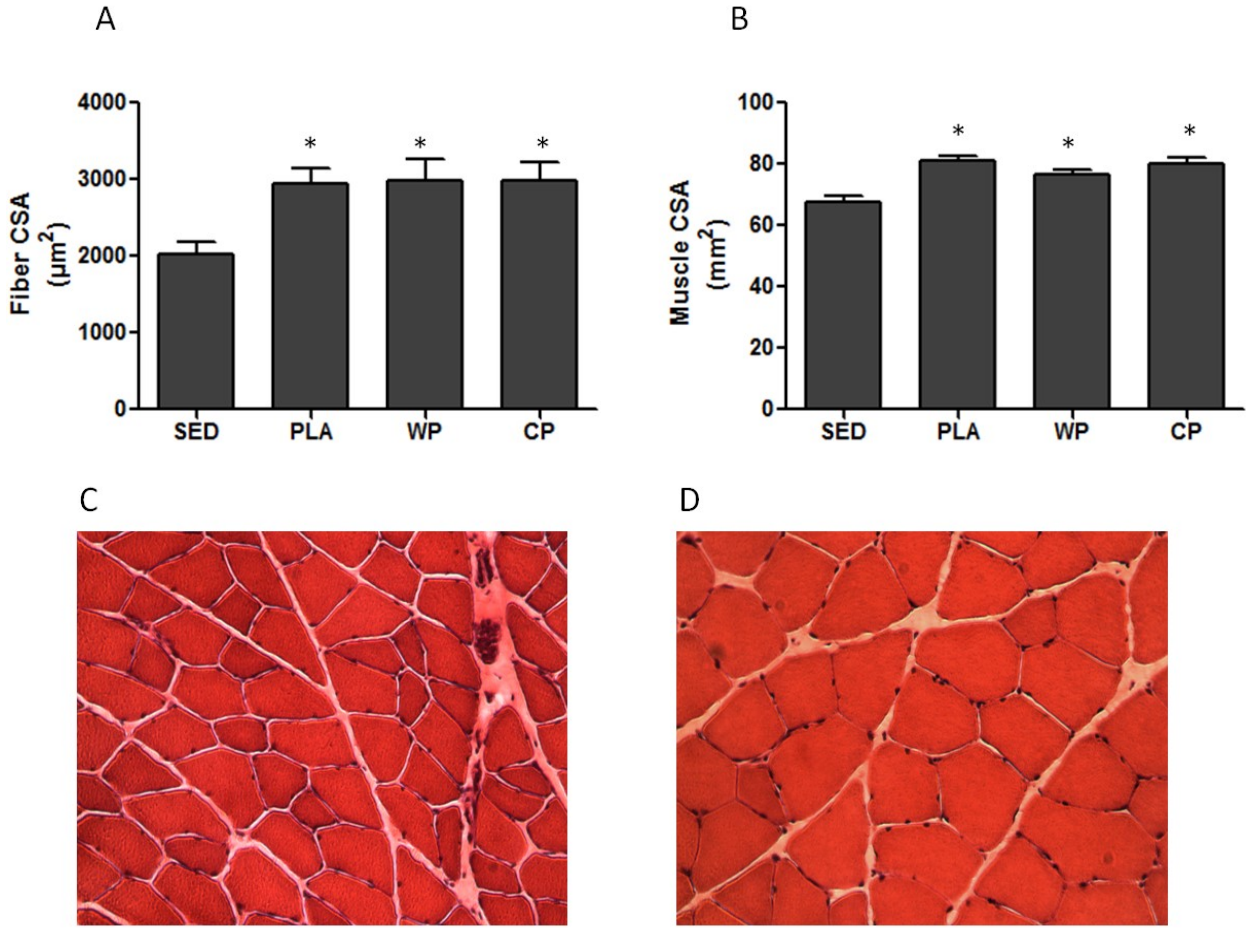

Figure 5.4. Cross sectional area**.** A) Fiber cross sectional area. B) FHL muscle cross sectional area. C) Cross section of FHL muscle in untrained rats (20 x objective lens) . D) Cross section of FHL muscle in trained rats (20 x objective lens). Data are presented as mean ± SEM (n = 7 - 8 per group). *, $p \leq 0.05$ vs. SED.

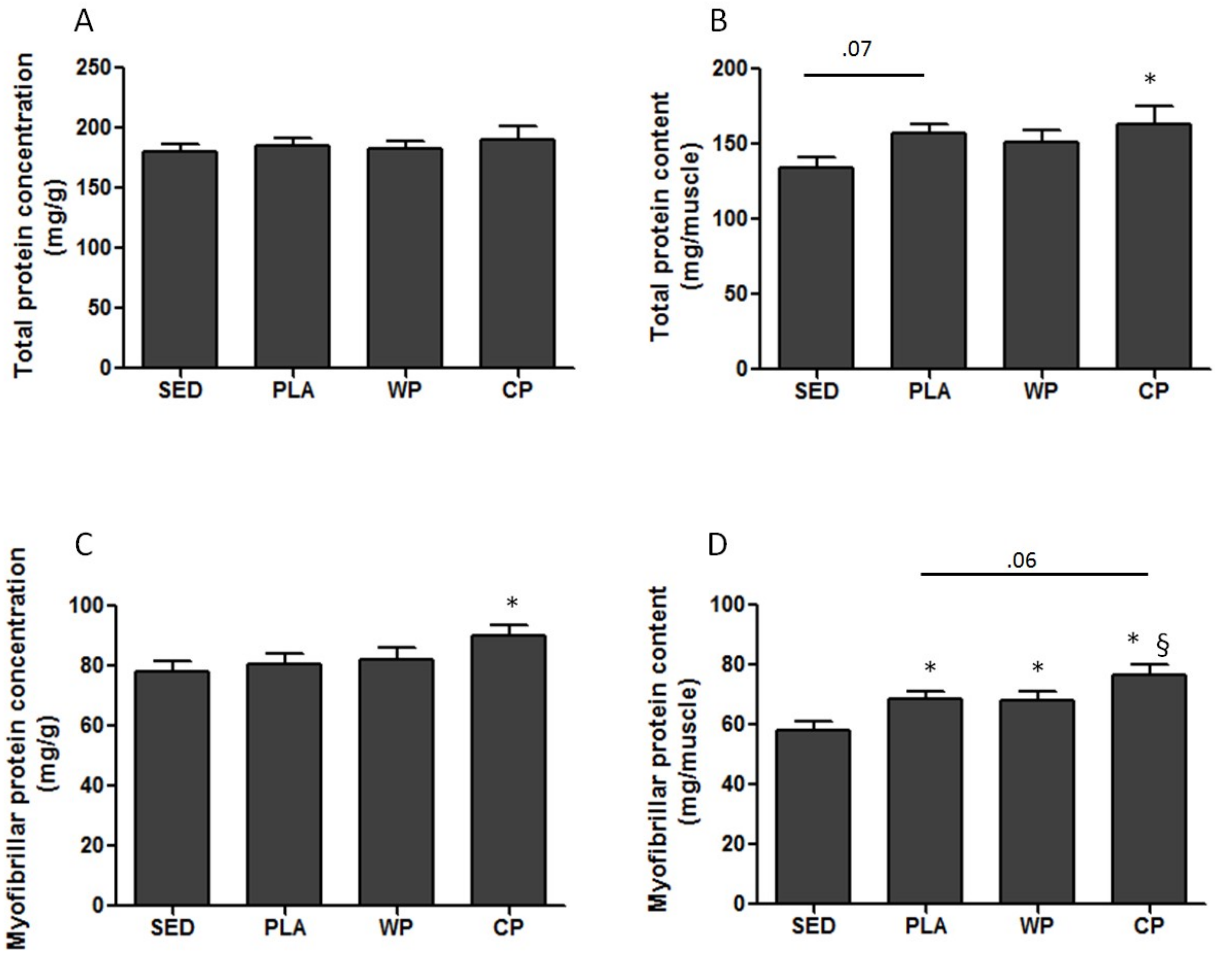

Figure 5.5. Total and myofibrillar proteins. A) Total protein concentration. B) Total protein content per muscle. C) Myofibrillar protein concentration. D) Myofibrillar protein content per muscle. Data are presented as mean ± SEM (n = 7 - 8 per group). *, $p \leq 0.05$ vs. SED. §, $p \leq 0.05$ vs. WP.

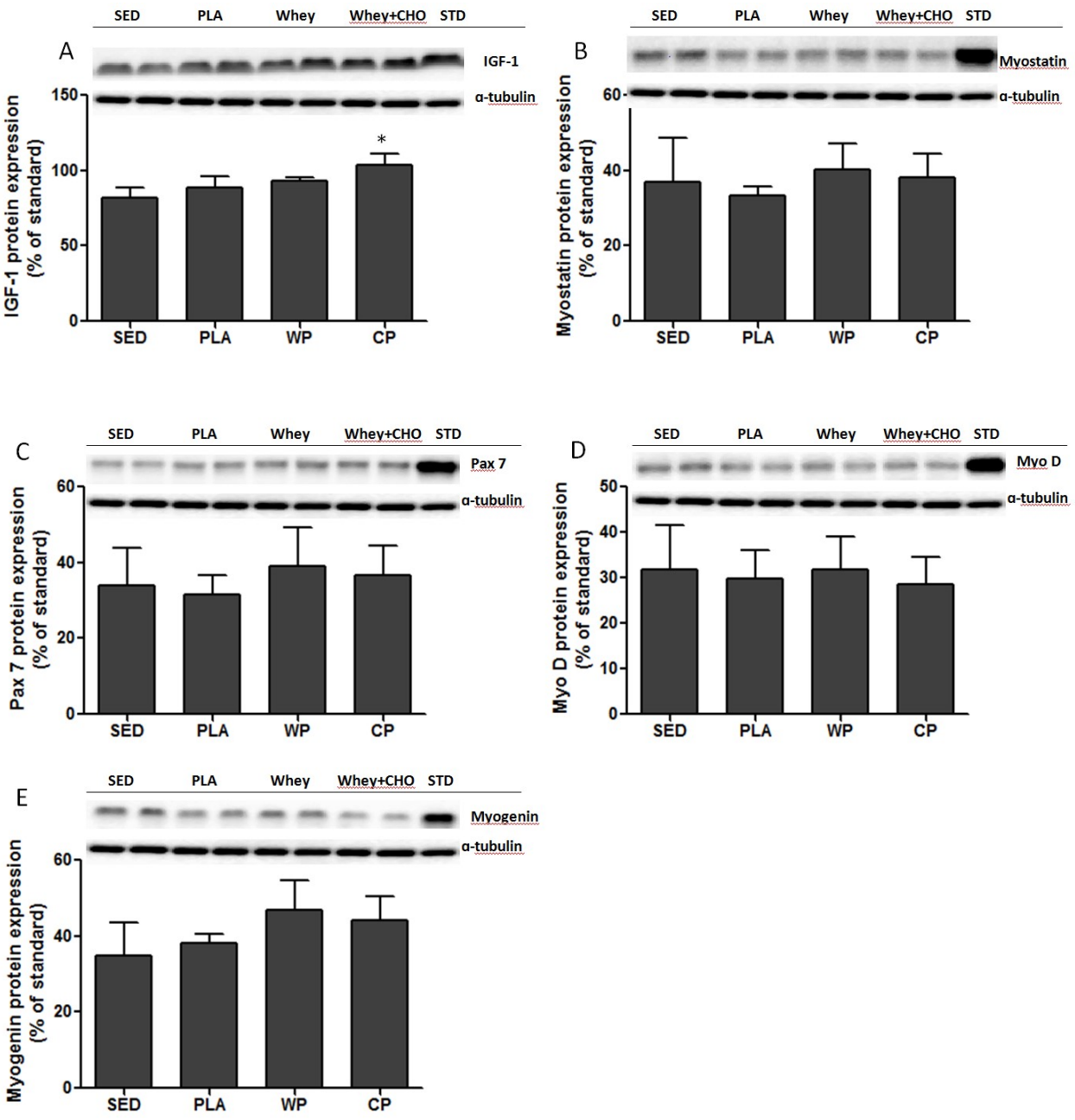

Figure 5.6. Markers for new nuclei involvement**.** A) IGF-1 protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. B) Myostatin protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. C) Pax 7 protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat

tissue. D) Myo D protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. E) Myogenin protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. Data are presented as mean ± SEM (n = 7 - 8 per group). *, $p \leq 0.05$ vs. SED.

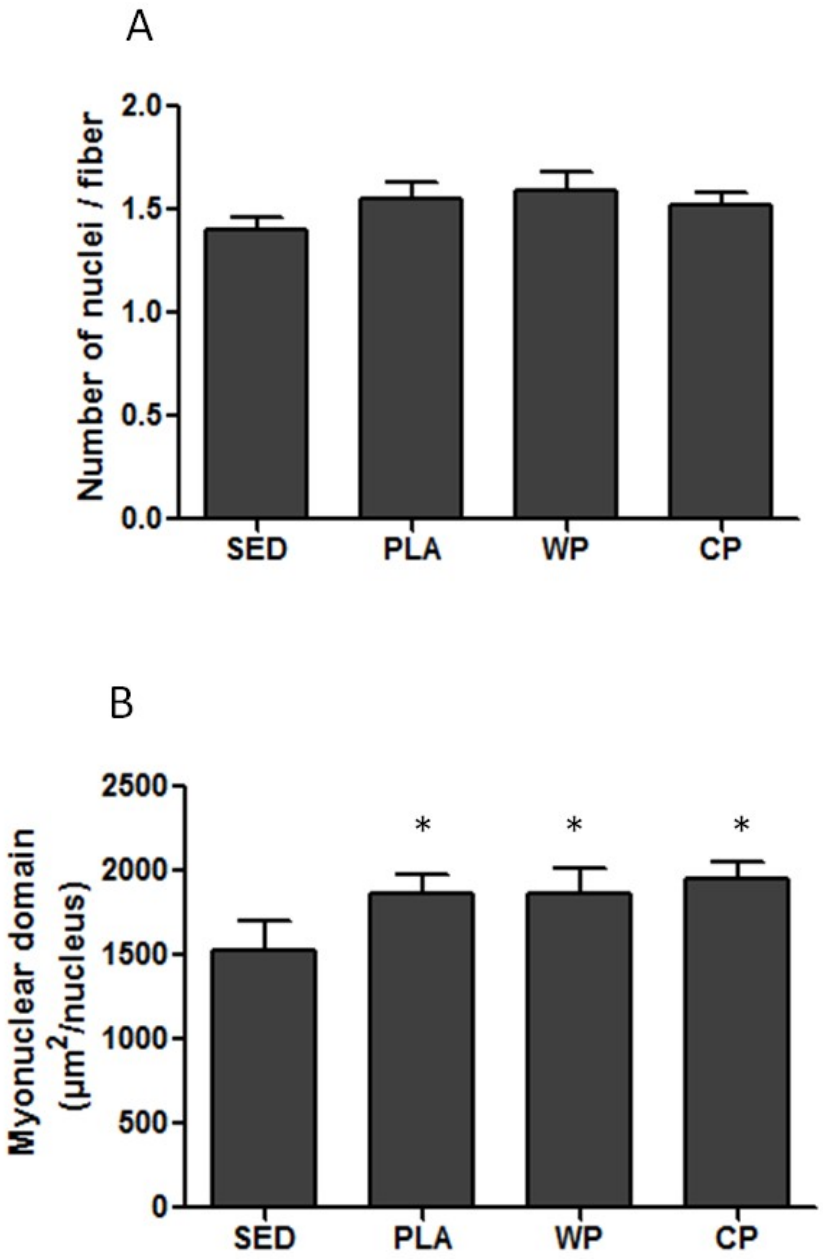

Figure 5.7. Number of nuclei and myonuclear domain**.** A) Number of nuclei per FHL muscle fiber. B) Myonuclear domain per nucleus. Data are presented as mean ± SEM (n = 7 - 8 per group). *, $p \leq 0.05$ vs. SED

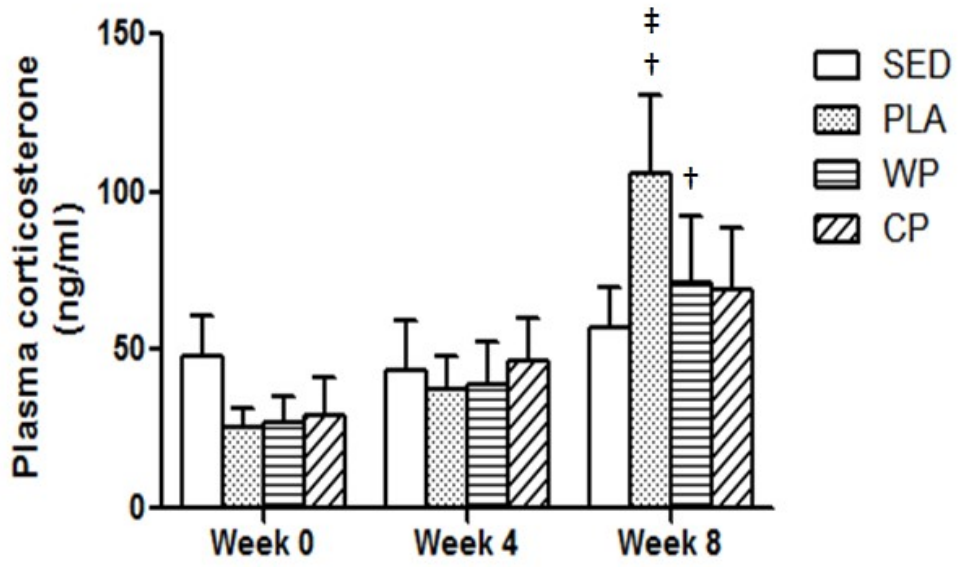

Figure 5.8. Plasma corticosterone level 22-24 hours after 1st (week 0), 10th (week 4), and 20th (week 8) exercise session. Data are presented as mean ± SEM (n = 7 - 8 per group). †, $p \leq 0.05$ vs. week 0 in the same treatment. ‡, $p \leq 0.05$ vs. week 4 in the same treatment.

Chapter VI: GENERAL DISCUSSION

The purpose of this series of studies was to determine the effect of carbohydrate (CHO) and amino acids (AAs) on signaling pathways that regulate muscle protein synthesis (MPS) and muscle protein breakdown (MPB) following acute resistance exercise (RE), and muscle adaptations following prolonged resistance training and the underlying mechanisms.

Two major strategies have been well documented to improve skeletal muscle adaptation: RE and feeding. Skeletal muscle is a plastic tissue that is highly responsive to mechanical loading (13). With respect to nutritional supplements, many investigations have reported that protein supplementation, in particular whey (WP), enhances MPS by ensuring sufficient essential AA and activating the mTOR signaling pathways (6, 19, 23, 26). However, not all AA in the composition of protein equally contribute to muscle accretion. Glutamine, a conditionally essential amino acid (EAA), has been reported to play an important role to regulate muscle protein turnover under different conditions, particularly under a catabolic state (17, 18, 25, 27). Compared with glutamine, however, AlaGln is more stable in solution, and produces a higher plasma glutamine concentration (1, 9, 16), but the effect of AlaGln on protein accretion was unknown. Therefore, in the first study, we compared the effectiveness of AlaGln and WP on signaling proteins that control MPS and MPB. The results showed that AlaGln may inhibit MPB via restraining the activation of AMPK-FOXO3A and NF-kB p65 post exercise, but it did not have any effect on signaling proteins related to MPS. We also found that WP appears to accelerate the activation of the mTOR signaling pathway, but this effect did not appear to be regulated by plasma hormonal circulation. Signaling proteins that control MPB were not influenced by the WP supplementation. Collectively, our findings suggest that a combination of WP and AlaGln supplementation provided post exercise may result in the phosphorylation of signaling proteins in a manner that increase MPS and decrease MPB.

In addition to the beneficial effect of protein/AA supplementation on muscle accretion, lines of evidence have demonstrated that CHO can reduce circulating the cortisol level after exercise (2, 20) and suppress MPB (5). Although CHO has been shown to significantly increase the plasma insulin level, the role of CHO on MPS and subsequently muscle hypertrophy remain inconsistent. Therefore, in studies 2 and 3, we investigated whether adding CHO to a protein

supplement can promote an increase in MPS after an acute resistance exercise, and enhance greater muscle hypertrophy and strength over 8 weeks of resistance training. In the second study, we found that post exercise supplementation with CHO plus protein (CP) hastened MPS during the early period of exercise recovery compared with placebo (PLA) or WP alone. With regard to the phosphorylation of signaling proteins that are involved in MPS and MPB, CP yielded a greater phosphorylation of mTOR than SED at 1 h post exercise, and SED and WP at 2 h post exercise. Because CP significantly elevated the plasma insulin level at 1 h post exercise, the increased MPS for CP appears to be associated with a greater insulin response. Both WP and CP increased 4E-BP1 phosphorylation relative to PLA at 1 h post exercise, and this phosphorylation level was higher for CP and WP than SED at 2 h. Earlier research suggested that reduced 4E-BP1 phosphorylation may result in the inhibition of protein synthesis during resistance exercise (7). Therefore, inhibition of 4E-BP1 by supplementation during early hours of exercise recovery might contribute to an enhancement of MPS.

Another signaling pathway mediating MPS is GSK3-eIF2Bε, and is independent of the mTOR signaling pathway. We observed no differences in the phosphorylation of Akt, GSK3, and eIF2Bε across time or treatment. These results implied that the regulation of MPS by the GSK3-eIF2Bε dependent signaling pathway occurs during the later stages of exercise recovery, which tends to agree with earlier research findings (4, 8, 12). In contrast, our results indicate that the activation of mTOR and the inhibition of 4E-BP1 by CP play important roles in the stimulation of MPS during the early phase of exercise recovery.

AMPK, an energy dependent kinase, has been shown to inhibit mTOR activation, which then suppresses MPS (3, 7). AMPK can also activate FOXO3A, which in turn stimulates MPB (24). An interesting finding was that exercise significantly elevated the phosphorylation of AMPK at 1 h post exercise, and this level remained elevated only in the WP at 2 h post exercise. It is possible that at 2 h post exercise, WP induced MPS but energy availability was insufficient resulting in an elevated ADP/ATP to activate AMPK. With regard to MPB, the phosphorylation of FOXO3A was significantly elevated by exercise at 1 h post exercise, and only the CP group showed sustained phosphorylation of FOXO3A relative to the SED group at 2 h post exercise. However, the change of FOXO3A did not follow the same pattern as AMPK. Furthermore, the plasma corticosterone level was elevated immediately after exercise, but returned to the basal

level at 1 h post exercise. Cortisol is a strong catabolic hormone stimulating MPB as well as inhibiting MPS (21, 22). In agreement with our findings, previous results from our laboratory showed that plasma cortisol did not differ within 6 h post exercise with or without CP provision, but plasma cortisol was elevated for PLA as compared with the CP group at 24 h post exercise (2). According to our FOXO3A and corticosterone results, it is unlikely that CP supplementation contributed to the inhibition of MPB during the early phase of exercise recovery. Together, it is concluded that CP accelerates MPS during the early recovery of resistance exercise relative to PLA and WP. This enhancement in MPS could have been the result of a rise in plasma insulin and the activation of the mTOR signaling pathway.

It was speculated that CP supplementation, which induces MPS after acute resistance exercise, might produce a greater training adaptation than PLA and WP during prolonged resistance training. Therefore, in the third study, we compared the effect of CP, WP, and PLA on muscle hypertrophy and muscle strength. Rats underwent progressive resistance exercise training every three days for 8 weeks. Nutritional supplementation was provided immediately after each exercise session. The primary finding was that maximal carrying capacity, indicative of muscle strength, was enhanced by 2.7-fold in both PLA and WP groups after 8 weeks of training, and the capacity was further increased by CP (3.04-fold) relative to the PLA and WP. This improvement in training performance for CP was likely to be due to the greater increase in total and myofibrillar protein content than in the PLA and WP groups. Myofibrillar protein contains contractile proteins, such as myosin and actin. The more myofibril is packed in the muscle fibers, the greater the muscle hypertrophy, and the stronger force the muscle can produce. It is worth noting that muscle and fiber cross sectional area (CSA) were significantly increased by exercise training, but there were no differences among exercise groups. Skeletal muscle is composed of various proteins, including myofibrillar protein, mitochondria, and sarcoplasm, and they demonstrate different responses to resistance exercise. We cannot rule out the possibility that CP brings about more myofibrillar synthesis with a compensatory decrease in other types of proteins in muscle. It is also possible that PLA and WP develop more muscle damage than CP, and consequently lead to muscle edema. This may explain the greater enhancement of muscle strength with CP supplementation, but with the same muscle CSA among exercise groups. The accumulation of myofibrillar protein results from increased MPS and decreased MPB. We found

that plasma corticosterone level was elevated in PLA and WP over 8 weeks of training, whereas corticosterone level did not change in the CP. As discussed in the second study, corticosterone is a strong catabolic hormone, leading to a negative nitrogen balance by increasing MPS and decreasing MPB. Low corticosterone level in the CP group may have attenuated MPB. Additionally, IGF-1 protein expression was upregulated in the CP group compared to the SED group. Skeletal muscle IGF-1 is capable of stimulating protein synthesis and satellite cell (SC) activation (14, 15). Therefore, CP appears to promote greater accumulation of myofibrillar protein, as compared with PLA and WP, by increasing IGF-1 and attenuating exercise induced elevation in corticosterone circulation.

Once protein accretion reaches a certain level, further enhancement in muscle hypertrophy may be accompanied by an increased number of myonuclei in order to maintain a efficient functioning myonuclear domain (area of cytoplasm per nuclei). It is believed that satellite cells (SC) are the sole source for providing new myonuclei. Myogenic regulatory factors (MRF) that are relevant to the recruitment of new nuclei were examined in the third study. Changes in Pax 7, MyoD, and myogenin were not observed across all treatment groups. We then measured the total number of nuclei per fiber and calculated the myonuclear domain. The amount of nuclei per fiber did not differ among groups, but exercise training did enlarge the myonuclear. Therefore, muscle hypertrophy induced by resistance training was not accompanied by an increase in myonuclei, indicating that the existing number of myonuclei were capable of supporting the enlargement of muscle fibers.

Resistance training also produced a greater increase in the FHL muscle and slight reduction in the epididymis adipose tissue. However, no differences were observed among the exercise groups. In addition, percent of fat and lean mass did not differ among treatment groups.

Taken together, these three studies suggest that nutritional supplementation provided immediately post exercise will enhance muscle protein accretion. In particular, AlaGln may inhibit MPB via phosphorylating AMPK-FOXO3A and NF-Kb p65 signaling pathways. Co-ingestion of CHO and WP accelerates MPS during the early recovery phase from acute resistance exercise relative to PLA and WP, and its cumulative effect during resistance training is to accelerate strength gains by increasing production of more myofibrillar protein per unit muscle.

FUTURE DIRECTIONS

This series of studies has demonstrated that protein/AAs and CHO supplementation are beneficial for acute resistance exercise recovery and prolonged training adaptation. These three studies also addressed the underlying signaling pathways that are involved in MPS, MPB, and muscle hypertrophy. Clearly, this is an area for more research to be performed in order to better understand the role of nutritional supplementation, in particular of CHO and WP, on exercise performance and training adaptation.

In the first study, we did not observe the effect of AlaGln on signaling proteins that regulate MPS. Perhaps the glutamine level did not fall below its normal physiological level during exercise; therefore its *de novo* synthesis was sufficient to maintain MPS. However, we did not measure the blood glutamine level directly. In the second study, we speculated that the beneficial effect of CHO on MPS and the mTOR signaling pathway might be due to a rise of insulin level, which then trigger more AA transported into the muscle fiber. However, we did not measure plasma and intracellular AA concentration directly. L-Leucine uptake is crucial to activation of the mTOR signaling pathway, so the measurement of intracellular leucine is also important. According to these limitations in studies 1 and 2, a future study could be designed to address the impact of AA concentration in plasma and muscle on MPS. Moreover, additional time course studies are needed to determine the effect of CHO and WP on MPS during the later stages of exercise recovery. Alternatively, an isotopic ($2H_2O$) methodology could be used to measure 36 h fractional rates of MPS to determine cumulative MPS (10, 11).

In the third study, we did not see an improvement in rat body composition (increase in lean mass percent and/or decrease in fat mass percent) after 8 weeks of resistance training. Only the FHL muscle demonstrates a hypertrophic response to ladder climbing, and hypertrophy of a single muscle is not adequate to observe changes in body composition. Therefore, other resistance exercise models, such as squat resistance exercise, should be considered for future resistance exercise studies. Furthermore, to better investigate CP and resistance training adaptations, other animal models, as well as different age ranges, need to be considered. Eventually, it will be necessary to confirm these findings in human research studies. Also, to fully examine how CP supplementation affects MPS, MPB, and training adaptation, we could

expand our investigations into various populations, such as elite athletes, adolescents, the elderly undergoing sarcopenia, and type 2 diabetics. CP supplementation could also be applied a bed rest population to determine its effect on muscle atrophy prevention. Applying CP supplementation to a wider population would broaden our understanding of how CP enhances/maintains muscle mass and strength, and improves quality of life.

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Appendices

APPENDIX A: BLOOD SUBSTRATE AND HORMONE MEASUREMENT

Glucose Assay

Plasma glucose was determined using a colorimetric method, which employs glucose oxidase (GOD) and a modified Trinder color reaction [1] (Sigma Chemical, St. Louis, MO). The modified Trinder reagent contains the enzymes peroxidase (HPOD), 4-aminoantipyrine (4-AAP) and p-hydroxybenzene sulfonate (p-HBS). GOD oxidizes glucose to D-gluconate and forms an equal amount of hydrogen peroxide. 4-AAP and p-HBS are oxidized by hydrogen peroxide and form a quinoneimine dye. The dye is intensely red in color. The absorbance of the reaction solution is measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 500 nm. The intensity of the color in the reaction solution is proportional to the glucose concentration in the sample. All samples were run in duplicate. Difference between duplicate results of a sample of < 7% CV was acceptable.

Three different concentrations of glucose were used as standards and controls to monitor the performance of assay procedures. Within and between assay variations was performed on the three standards containing varying concentrations of glucose.

Sample No.	Mean mg/dl	Within % CV	Between % CV
1	50	1.9	9.3
2	100	3.7	7.3
3	200	3.8	2.1

Lactate Assay

Blood lactate was determined from the PCA extract according to the protocol of Hohorst [2]. Coupled with β-NAD and hydrazine, lactate can be converted to pyruvate and generate NADH by lactate dehydrogenase (LDH). The amount of lactate is then determined by the generation of NADH using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc.,

Fullerton, CA) at a wavelength of 340nm. All samples were measured in duplicate. Difference between duplicate results of a sample of < 10% CV was acceptable.

Growth Hormone Assay

Plasma growth hormone was measured using sandwich ELISA kit with CV < 10% (Millipore Corporation, MA). The growth hormone from samples is captured by a pre-titered amount of anti-growth hormone polyclonal antibodies coated in a microtiter plate. It is then recognized by a secondary polyclonal antibody, which is conjugated with horseradish peroxidase. The quantification of the conjugation is visualized at dual wavelengths of 450nm and 630nm.

Insulin-Like Growth Factor 1 (IGF-1) Assay

Plasma IGF-1 was determined using a high-sensitivity ELISA kit with CV <10% (Immunodiagnostic Systems Inc., AZ). Samples were pretreated prior to assay to separate the IGF-1 from the IGFBPs, and then diluted for assay. A monoclonal anti-mouse IGF-1 is coated onto the inner surface of microtitre wells. The diluted samples are incubated with biotin labeled polyclonal goat anti-mouse IGF-1 for 1 h at room temperature. Horseradish peroxidase labeled avidin is added to bind to biotin, followed by a wash step. Color is developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures are read at a wavelength of 450nm (reference 630nm).

Corticosterone Assay

The concentration of corticosterone was determined by an enzyme-linked immunosorbant assay kit (ELISA) (Enzo life sciences Inc. Ann Arbor, MI. Cat ADI-900-097). This kit is a competitive immunoassay. Plasma samples were diluted (1:40). In brief, 10µl of each sample was added into microfuge tube. Then, 10 µl 1:100 diluted steroid displacement reagent (SDR) solution was added to each sample tube, and vortex. The sample tube then stayed at room temperature for 5 min. 380 µl ELISA assay buffer was added to each tube and vortex. Then, 100 µl of standard diluents was added into the NSB and the Bo (0pg/ml standartd) wells. After

dilution, 100 µl of standard and or samples was added into the appropriate wells. A polyclonal antibody was added into the well which bound to corticosterone in the standard or sample tubes or an alkaline phosphoatase molecule which has corticosterone covalently attached to it. After incubation, the enzyme reaction was stopped and each well read on a microplate reader at 405nm.

Rat Insulin Radioimmunoassay (RIA)

Rat plasma insulin was determined using an RIA method (#RI-13K, Millipore, Millipore Corporation, MA). ^{125}I-labeled insulin was added to glass tubes (12 x 75 mm) containing standards, controls, or plasma samples. Rat insulin antibody was added to the tubes containing standards or samples. All tubes were incubated at 4°C overnight (20-24 hours). During that time, ^{125}I-labeled and unlabeled insulin competed for binding sites on the antibody. Thus, the amount of tracer bound to antibody was decreased as the concentration of unlabeled antigen increased. The next day, the antigen-antibody complex was precipitated by adding precipitating solution, incubating the tubes, and centrifugation. Once the supernatant was poured off, the tubes were counted for 2 min in a gamma counter (Perkin Elmer life sciences, Turku, Finland). A standard curve was generated by the counter and used to calculate sample concentration. In studies 1 and 2, the insulin in plasma samples was measured in duplicate. If the difference between duplicates was greater than 10% CV, the sample was reanalyzed. The fasting level of insulin in the rat is generally in a range of 0.5-2.0 ng/ml.

APPENDIX B: MUSCLE HOMOGENIZATION

Muscle homogenization buffer (100ml, pH7.4)

Reagent	Concentration (mM)	Amount (g)
HEPES	20	0.477
EGTA	2	0.076
NaF	50	0.209
KCl	100	0.746
EDTA	0.2	0.0074
β-Glycerophosphate	50	1.08
DTT	1	0.015
PMSF	0.1	0.00174
Benzamidine	1	0.0157
Soduim vanadate	0.5	0.0092

Add reagents in order to 80ml ddH_2O, adjust pH to 7.4, and bring the volume to 100ml with stirring on a magnetic plate.

Homogenization procedure

1. Weigh approximately 80-100 mg of muscle sample.
2. Dilute muscle to 1:8 (wt/vol) in homogenization buffer.
3. Homogenize muscle on ice (3 x 10sec strokes) with a glass tissue grinder pestle at 3,000 rpm by an electrically-powered stirrer (Corning Life Sciences, Lowell, MA).
4. Centrifuge muscle samples at 14,000 g for 10 minutes at 4°C.
5. Aliquot the supernatant into 1.5ml test tubes and store them at -80°C.

APPENDIX C: WESTERN BLOT

Solutions

30% Acrylamide and 1% Bisacrylamide Mixture (200ml)

Add 58g Acrylamide and 2g Bisacrylamide in order to 150ml ddH_2O while stirring on a magnetic plate, and bring to 200ml volume with ddH_2O. Filter this mixed solution through Whatman #1 filter paper and stored in a dark bottle due to the high light sensitivity at 4°C.

1.5M Tris, pH8.8 (500ml)

Add 90.82g Trisbase to 400ml ddH_2O while stirring on a magnetic plate and bring to 500ml volume with ddH_2O. Adjust pH to 8.8 with 12N and 1N HCl. Store it at 4°C.

1.0M Tris, pH 6.8 (500ml)

Add 60.57g Trisbase to 400ml ddH_2O while stirring on a magnetic plate and bring to 500ml volume with ddH_2O. Adjust pH to 6.8 with 12N and 1N HCl. Store it at 4°C.

10% SDS (100ml)

Add 10g SDS to 80ml ddH_2O while stirring on a magnetic plate and bring to 100ml volume with ddH_2O. Filter this solution through Whatman #1 filter paper.

20% SDS (100ml)

Add 20g SDS to 80ml ddH_2O while stirring on a magnetic plate and bring to 100ml volume with ddH_2O. Filter this solution through Whatman #1 filter paper.

10% APS (1ml)

Add 0.1g APS to 1.5ml test tube with 1ml ddH_2O and vortex until dissolve. Make fresh daily.

10 x Running Buffer (2L)

Reagent	Amount (g)
Trisbase	60.56
Glycine	288.4
SDS	20g

Add the reagents into 1.5L ddH_2O stirring on a magnetic plate and bring to the volume with ddH_2O

10x TTBS (2L, pH7.4)

Reagent	Amount
TrisBase	24.2 g
NaCl	175.36 g
Tween 20	12 ml

Add the reagents into 1.5L ddH_2O while stirring on magnetic plate and bring to the volume

Ponceau S.

Reagent	Amount
Dye-Ponceau S	0.05 g
Acetic Acid	2.5ml

Add the reagents into 50ml ddH_2O and vortex until dissolve. Recycled to be used later.

Wet-transfer Buffer (2L for 2 tanks)

Reagent	Amount
Glycine	28.8g
Trisbase	6.04g
Methanol	300ml

SDS	0.75g

Add reagents in order into 1400ml ddH_2O while stirring on a magnetic plate. Bring the volume into 2L with ddH_2O.

0.25% Bromophenol Bule

Add 0.0125g Bromophenol Bule into 5ml ddH_2O

Sample buffer (50ml)

Reagent	Amount
1.25M Tris-HCl (Ph 6.8)	5ml
Glycerol	10ml
20% SDS	5ml
B-Mercaptoethanol	2.25ml
0.25% Bromophenol Blue	1.6ml

Add reagents in the order listed to 20ml ddH_2O while stirring on a magnetic plate and bring to volume with ddH_2O.

Western blot procedure

1. Preparing resolving gel solution (10% and 15% resolving gel for 2 gels).

	10% gel	**15% gel**
Reagent	**Amount**	**Amount**
H_2O	3.2 ml	1.8 ml
Acrylanide mix	2.67 ml	4 ml
1.5M Tris (pH 8.8)	2 ml	2 ml
20% SDS	0.08 ml	0.08 ml

10% APS	0.08 ml	0.08 ml
TEMED	0.008 ml	0.008 ml

Add all reagents above into 50ml test tube. Then mix them and fill ¾ full caster with a glass pipette. Overlay with 300ul butanol. Allow 40 min for gel to polymerize.

2. After gel polymerization, pour off butanol from the resolving gel and rinse between casting plates with ddH_2O and dry it with KimWipes.

3. Preparing stacking gel solution (for 2 gels).

Reagent	**Amount**
H_2O	3.4 ml
Acrylanide mix	0.83 ml
1.0M Tris (pH 6.8)	0.63 ml
20% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

Add all reagents above into 15ml test tube. Then mix them and fill the caster with stacking gel solution then put combs into place. Allow 45 min for stacking gel to polymerize.

4. Preparing samples.

a. Take the samples out of -80°C and thaw them on ice;
b. Dilute samples 1:2 with sample buffer in labeled microcentrifuge tubes;
c. Vortex and place in boiling water (~90-95°C) for 10 min.

5. Preparing 1 x running buffer.

Add 100ml 10x running buffer into 900ml ddH_2O stirring on a magnetic plate.

6. After stacking gel has polymerized, carefully remove the combs and assemble gel apparatus. Fill inner chamber and outer chamber with 1 x running buffer.

7. Load 12 ul of standard, 12-20 ul of sample, or 8 ul of molecular weight marker to corresponded gel lane.

8. Electrophoresis at 100-135 V for 60-90 min or 90 V for 150 min.
9. After the electrophoresis, prepare for wet-transfer buffer showed as above.
10. Cut 9 x 6 cm rectangle Nitric Cellular Membrane, label it, and place membrane in wet-transfer buffer for 5 min on a shaker.
11. Carefully separate casing plates, cut off stacking gel and place resolving gel in wet-transfer buffer for 5 min on a shaker.
12. Soak four of the spongy pads and filter paper (2 for each gel) in the wet-transfer buffer for a few minutes.
13. Making the gel "sandwich".

a. Place cassette in a container filled with transfer buffer. Black side down;

From bottom to top, add a spongy pad, a filter paper, a gel, a NC membrane, another filter paper, another spongy pad. Roll out bubbles with wetted glass tube.

b. Close cassette. Place in transfer apparatus;
c. Add ice to the small container and place in transfer apparatus as well;
d. Fill the transfer apparatus with transfer buffer and add the top piece with electrodes to the apparatus;
e. Place transfer apparatus in a larger plastic container and fill the large plastic container with ice;
f. Electrophoresis at 90V for 45 - 90 min.

14. During the transfer, prepare 1 x TTBS.

Add 100ml 10 x TTBS into 900ml ddH_2O while stirring on a magnetic plate.

15. Prepare 7% non-fat dry milk (NFDM) - 30ml per membrane.

Add 4.2g NFDM into a 100ml beaker containing 60ml 1 x TTBS while stirring on a magnetic plate.

16. After the transfer, take out the "sandwich". Stain each NC membrane with Ponceau S. for 5 seconds and put membrane into 1 x TTBS on a shaker to wash out Ponceau S. on Membrane.

17. Place NC membrane in a container with 7% NFDM and block for 20 min with gentle agitation.

18. During blocking, prepare antibody with 2% NFDM. 10ml per membrane.

    Add 0.4g NFDM into 20ml 1 x TTBS stirring on a magnetic plate. Then add appropriate ratio of antibody into 2% NFDM.

19. After blocking the membrane, wash 5 min in 25ml TTBS.

20. Incubate membrane with the primary antibody overnight at 4 °C with gentle agitation.

21. After overnight incubation with the primary antibody, wash membrane 3 x 5 min in 25 ml TTBS.

22. Incubate membrane with species specific secondary antibody for 1 h at room temperature with gentle agitation.

23. After incubation with the secondary antibody, wash membrane 3 x 5 min in 25 ml TTBS.

24. Visualize protein bands using an ECL detection kit and Bio-Rad ChemiDoc detection system according to the manufacturer's instructions.

25. Quantify the density of bands using Quantity One Analysis software.

Membrane stripping and re-probing

1. Wash membrane in the ddH_2O for 5 min.
2. Add membrane to 0.2N NaOH for 5-7 min.
3. Wash membrane in the ddH_2O for 5 min, followed by another 5 min wash using TTBS.
4. After washing, membrane can be re-blocked and then re-probed with another antibody.

APPENDIX D: LOWRY PROTEIN ASSAY

1. Thaw one aliquot of 5mg/ml bovine serum albumin (BSA) stock (-20°C) and serially dilute it with deionized water to generate a standard curve as follows.

	Water (ml)	Protein (ml)	Protein Conc.(mg/ml)
Blank	1.0	0	0
A	1.8	0.2 of stock	0.5
B	0.2	0.8 of A	0.4
C	0.5	0.5 of B	0.2
D	0.5	0.5 of C	0.1
E	0.5	0.5 of D	0.05
F	0.5	0.5 of E	0.025

Set up duplicate 12x75mm glass assay tubes of each standard.

2. Dilute samples with deionized water in 1:30 ratio. Keep on ice. Set up duplicate glass assay tubes for each sample.

3. Make Solution A: add 48 ml of sodium carbonate (20 g/l of 0.1 NaOH) to a 125 ml Erlenmeyer flask. Gently layer 1 ml of 2% sodium potassium tartate over the surface of the sodium carbonate. Then gently layer 1 ml of 1% cupric sulfate over the sodium potassium tartate. Mix Solution A by swirling, then by vigorously vortexing.

4. To the duplicate 12x75mm glass assay tubes, add 0.1 ml of standards or samples. Add 1.0 ml of Solution A to each tube. Next vortex each individual tube. Incubate tubes at room temperature for 10 minutes.

5. Dilute phenol reagent 1:2 with deionized water. After the tubes have incubated for 10 minutes, add 0.1 ml of the diluted phenol reagent into each glass tube while vortexing the glass tube. Incubate at room temperature for 30 minutes.

6. Turn on the spectrophotometer and let the bulb warm up at a wavelength of 750 nm. Once the 30 min incubation period is complete, blank the spectrophotometer with the “blank” tube. Then read every sample. Use the standard curve to determine protein concentration of the samples.

APPENDIX E: MUSCLE PROTEIN SYNTHESIS MEASUREMENT

SUnSET

Surface Sensing of Translation (SUnSET) is a non-radioactive technique used in the measurement of MPS. Puromycin is a structural analog of aminoacyl-transfer RNA (tRNA). Due to its special structure, puromycin can compete with tRNA and be incorporated into the nascent polypeptide chain, which then terminates the peptide elongation. Puromycin-labeled peptide can be detected using Western blotting and reflect changes in muscle protein synthesis. In our study, 100 mg puromycin (puromycin-DiHCl: PJ593 Bio Basic, Markham, Ontario, Canada) is dissolved in 10 ml phosphate buffered saline (PBS) solution (pH 7.4). 30 min before muscle extraction, 0.04μmol/g body weight puromycin is given via intraperitoneal injection. Rats then are put back into their respective cages. Around 15 min after puromycin injection, rats are anesthetized using a combination of ketamine (80mg/kg) and xylazine (8mg/kg) so to assure that rats are unconscious entirely and ready for muscle dissection at exactly 30 min after puromycin injection.

Free Puromycin Measurement

Free puromycin as a precursor can be delivered to the muscle and incorporated into nascent peptide chains. The measurement of free puromycin was used to normalize the value of MPS obtained from western blot analysis in the same sample. The analysis was conducted as described previously [3] with slight modifications. Approximately, 30 mg of muscle were homogenized in ice-cold homogenization buffer at a 1:15 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). A 250μl aliquot sample homogenate was precipitated with 28μl of 100% trichloroacetic acid and incubated for 30 min on ice followed by 5 min of centrifuge at 4,200g. This was followed by the addition of 15μl of Tris buffer containing 1M Tris, 3M NaCl, and 1% Tween 20 at pH 7.0 and 30μl of 5.25M NaOH to 250μl supernatant to adjust the pH to ~9.0 (8.97-9.03). Next, the samples were filtered through a >3kDa filter (Amicon Ultra-0.5ml; Millipore, Carrigtwohill, Ireland) at 14,000g for 60 min. Meanwhile, a range of standards (0-40 pmol/100μl) were made and adjusted to pH 9.0. A 100μl sample or standard was added to a 96-

well amine-binding maleic anhydride activated plate (Pierce; Thermo Fisher Scientific) in duplicate and rocked overnight at 4ºC. The next day, the plate was washed 4 times using PBS with 1% Tween 20 (PBST with pH7.0) and blocked with 1% BSA-PBST for 45 min at room temperature. 100µl of anti-puromycin antibody (clone 12D10, 1:38,400) was added to each well and incubate for 105 min at RT. Following this incubation period, the wells were washed 3 times. Next, horseradish peroxidase-conjugated anti-mouse IgG Fc 2a (1:10,000; Millipore) was added to each well and incubated for 45 min at RT. After another 4 washes, Ultra 3,3',5,5'-tetramethylbenzidine (TMB, Thermo Fisher) was added to each well and rocked for 15 min. The reaction was then stopped by addition of 0.16 M sulfuric acid (Thermo Fisher). The absorbance was measured on a plate reader at a wavelength of 450nm. The concentration of free puromycin was calculated from a standard curve.

APPENDIX F: DETERMINATION OF FIBER CROSS SECTIONAL AREA

Tissue freezing

1. Excise fresh muscle tissue.
2. Place the distal half part of tissue in the cryo-mold submerging completely in the OCT compound.
3. Drop it immediately into nitrogen-cooled 2-methylbutane for 1 min.
4. Quickly wrap frozen tissue into a labeled aluminum foil and transfer into a freezer set at -80°C for later sectioning.

Tissue sectioning

1. Set the cryostat "specimen head" temperature to -20°C (Leica cm1900; Leica Microsystems Inc., Buffalo Grove, IL).
2. Transport frozen tissue into the cryostat chamber.
3. Mount tissue onto "specimen disks" using OCT compound, and insert the disk into "specimen head".
4. Adjust the blade holder and bring blade close to tissue.
5. Transversely section frozen tissue into 10µm on a cryostat.
6. Gently touch glass slide to sectioned tissue

Hematoxylin and Eosin (H & E) Staining

Frozen OCT embedded muscle was transversely sectioned into 10µm section on a cryostat (Leica cm1900; Leica Microsystems Inc., Buffalo Grove, IL) and mounted on a glass slide. Hematoxylin and eosin (H&E) staining (Fisher scientific Inc.) was performed to identify fiber CSA and quantify the number of myonuclei. 5 slides were put in a jar filled with Harris Hematoxylin for 5 min, followed by rinsing slides using tap water gently until water runs clear. Then, slides were put in a jar filled with Eosin for 2 min, followed by rinsing slides gently using tap water until water runs clear. Next, slides were rinsed by 70% ethanol, 100% ethanol, and xylene for 2-3 seconds, respectively in sequence so that the tissue could be dehydrated and hydrophobic. Stained slides were dried in the hood and covered by coverslip. Permount.

Hematoxylin stained nucleic acids to a deep blue-purple color, while eosin stained proteins nonspecifically with a pink color. Slides were observed with a light microscope (Nikon Diaphot, Nikon Corp.; Tokyo, Japan) with a 20x objective lens. Images were then taken using a mounted digital camera. Muscle fiber CSA and the number of nuclei were measured and counted using Image J software.

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APPENDIX G: FLOW CHARTS FOR EXPERIMENTS

Study 1

Rats (Sprague Dawley rats; N=89) housed in cages

(2 rats/cage)

↓

One-week acclimation

↓

Repeated sessions of ladder climbing every other day for 3 days without weight

(12 rats in sedentary groups stayed on the top of ladder for 20 min)

↓

3 practice sessions of climbing separated by 1 day with 50, 60, 70% of their weight, respectively

Attempt to climb to the top 8 times each practice session with a 2-min rest between climbs

(12 rats in sedentary groups stayed on the top of ladder for 20 min)

↓

Rats fasted on the testing day for 2 hours

↓

Start experiment

↓

Climb to the top of the ladder 10 times carrying 75% of their body weight with a 2-min rest between climbs

(12 rats in sedentary groups stayed on the top of ladder for 20 min)

↓

Rats were wrapped in a towel, and the tips of their tails cut and bled

↓

Blood sampling (0.7 ml)

↓

Rats were gavaged (8 ml/kg) with treatment solutions

↓

Immediately following either the 20 min or 40 min post intubation period, a drop of blood from the tail (0.7 ml) was collected

↓

Rats anesthetized IP (Na pentobarbital, 75 mg/kg)

↓

The Flexor Hallucis Longus (FHL) muscles were surgically excised

↓

Muscle samples were freeze-clamped and stored @ -80 °C until analyzed

↓

Rats were euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight), wrapped in paper and stored in the freezer until picked up by the Safety Office.

Measurements:

- Blood: glucose, insulin, GH, IGF-1, and lactate
- Muscles: p-mTOR, p-p70S6k, p-rpS6, p-Akt, p-FOXO3A, p-AMPK, and p-NF-kB p65

Study 2

After familiarization,

2-3 month old male Sprague-Dawley rats were fasted overnight (12h)

↓

Climbed to the top of the ladder 10 times carrying 75% of their body weight with a 2-min rest between climbs

(20 rats in sedentary groups stayed on the top of ladder for 20 min)

↓

Blood sampling (0.7 ml)

↓

Rats were gavaged (8 ml/kg) with treatment solutions

↓

0.04μmol/g puromycin was injected via IP 30 min before muscle extraction

↓

Rats anesthetized IP injection with a combination of ketamine (80mg/kg) and xylazine (8mg/kg) (15min after injection)

↓

Blood sampling (0.7 ml)

Excised the flexor hallucis longus (FHL) at 1 or 2 hours after gavage

(exacted 30 min after puromycin injection)

↓

Freeze clamped muscle samples and stored @ -80 °C until analyzed

↓

Rats were euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight). Wrapped in paper and stored in freezer until picked up by the Safety Office.

Measurements:

- Blood: glucose, insulin, corticosterone, GH, IGF-1
- Muscles: MPS, p-mTOR, p-p70S6k, p-rpS6, p-4E-BP1, p-Akt, p-GSK3α/β, p-eIF2Bε, p-FOXO3A and p-AMPK

Study 3

2-3 month old male Sprague-Dawley rats

↓

The day before resistance training started:

After 3 h (2-5pm) fast, rats were anesthetized IP (a combination of ketamine (70 mg/kg) and xylazine (7 mg/kg)) followed by the 1st body composition measurement by DEXA

↓

8 weeks resistance training: every 3 days

The initial exercise session: Food was deprived 3 h before exercise session

75% of body weight attached to the tail, +30g, +30g, … until maximal carrying capacity.

Blood sampling (0.7 ml)

Intubate different nutritional supplements

Food was provided 3 h after exercise session

↓

The subsequent training sessions: Food was deprived 3 h before exercise session, 50%, 75%, 90%, and 100% load of their previous maximal carrying capacity, +30g, +30g,… until maximal carrying capacity was determined

Intubate different nutritional supplementations

Food was provided 3 h after exercise session

↓

2nd and 3rd DEXA the day after 9th and 19th exercise session:

Food was deprived 3 h before exercise session 50%, 75%, 90%, and 100% load of their previous maximal carrying capacity, +30g, +30g,… until maximal carrying capacity was determined

Blood sampling (0.7 ml)

Intubate different nutritional supplementations

Rats anesthetized IP (a combination of ketamine and xylazine) 3 h after exercise session

2nd and 3rd body composition measurement by DEXA

Food was then provided after DEXA

↓

Sacrificing day:

21 h after the last exercise session, rats were fasted for 3 h

Rats anesthetized IP injection of a combination of ketamine and xylazine

The FHL on right side was excised and freeze clamped in liquid nitrogen. Distal half of the FHL on left side was embedded in OCT medium

Rats were euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight). Wrapped in paper and stored in freezer until picked up by the Safety Office.

- Groups: ex-PLA, ex-WP, ex-CP, SED
- Measurements

- ✓ Blood **(24 h after 1st, 10th, and 20th exercise sessions):** corticosterone
- ✓ Body weight measurement on the exercise day
- ✓ Muscle hypertrophy:
  - Muscle weight in the FHL muscle
  - Body composition: percentage of lean muscle mass and fat mass, absolute value of lean and fat mass using DEXA
  - Muscle cross section area (CSA) and mean fiber CSA
  - Total and myofibrillar proteins
- ✓ Muscle strength was determined by maximal carrying capacity
- ✓ The activation of SC
  - Indicators: Pax 7/Myo D/ Myogenin protein expression
  - Hormonal expression in muscle: IGF-1 and myostatin
  - Quantification of myonuclei and myonuclear domain

APPENDIX H: RAW DATA FOR STUDY 1

Study 1	Blood lactate (mM)						
20 min				40 min			
Rat #	Group	0 min	20 min	Rat #	Group	0 min	40 min
2	SED	1.65	1.08	11	SED	1.22	1.64
23	SED	1.30	2.18	21	SED	1.90	1.65
26	SED	1.07	1.45	22	SED	1.40	1.24
37	SED	0.64	0.74	26	SED	1.10	0.91
42	SED	0.98	0.42	35	SED	1.7	1.78
50	SED	0.52	1.00	38	SED	1.03	1.16
15	PLA	4.80	1.27	4	PLA	1.44	1.06
17	PLA	6.56	1.82	12	PLA	4.35	1.55
24	PLA	2.50	1.23	20	PLA	3.94	1.64
29	PLA	3.81	1.38	27	PLA	2.27	1.57
35	PLA	2.78	1.18	28	PLA	3.35	0.88
40	PLA	3.55	1.40	42	PLA	3.21	1.25
44	PLA	2.84	0.94	44	PLA	3.06	1.24
48	PLA	2.58	1.01	45	PLA	1.69	1.38
22	WP	2.18	1.29	13	PLA	2.54	1.24
25	WP	1.58	1.18	20	PLA	1.88	2.28
31	WP	1.56	0.66	6	PLA	3.91	1.10
33	WP	1.41	1.37	7	PLA	2.32	1.46
34	WP	2.84	1.45	13	WP	4.99	1.37
45	WP	2.89	2.64	19	WP	3.92	1.14
46	WP	3.89	1.58	24	WP	3.23	0.91
47	WP	2.43	0.67	29	WP	2.86	1.14
1	LSUS	5.22	1.88	32	WP	1.45	1.05
10	LSUS	3.30	1.82	34	WP	2.34	2.47
18	LSUS	3.15	1.56	46	WP	1.39	1.08
21	LSUS	2.13	0.94	18	WP	1.54	1.25
28	LSUS	2.67	1.31	4	WP	3.40	1.01
36	LSUS	2.87	0.69	19	WP	3.01	1.30
43	LSUS	1.74	0.79	8	WP	2.98	1.25
52	LSUS	2.52	0.58	11	WP	2.95	1.07
9	HSUS	2.95	1.73	3	LSUS	2.83	1.10
16	HSUS	3.39	2.57	10	LSUS	5.04	1.37
27	HSUS	2.06	1.97	14	LSUS	2.28	0.75
30	HSUS	1.66	0.84	16	LSUS	3.41	1.98
32	HSUS	2.68	1.16	17	LSUS	2.33	1.79
38	HSUS	3.38	1.68	30	LSUS	3.05	0.99
39	HSUS	2.48	1.04	39	LSUS	2.32	0.97
41	HSUS	2.86	1.73	41	LSUS	3.21	1.1
				8	HSUS	2.28	1.86
				9	HSUS	3.18	1.28
				15	HSUS	2.06	1.24
				23	HSUS	2.35	1.39
				25	HSUS	2.29	1.51
				31	HSUS	1.83	1.25
				33	HSUS	4.20	2.01
				40	HSUS	2.65	1.77
				3	HSUS	2.21	1.10
				14	HSUS	2.59	1.32
				5	HSUS	3.18	1.29
				12	HSUS	2.24	1.03

Study 1	Plasma glucose (mM)							
20 min					40 min			
Rat #	Group	0 min	20 min		Rat #	Group	0 min	40 min
2	SED	5.18	4.58		11	SED	6.87	6.50
23	SED	6.37	6.87		21	SED	6.72	6.57
26	SED	5.99	5.62		22	SED	8.41	7.32
37	SED	5.39	5.69		26	SED	6.62	6.54
42	SED	5.89	4.84		35	SED	6.96	6.13
50	SED	4.53	4.67		38	SED	5.55	5.78
15	PLA	6.40	5.17		4	PLA	7.65	6.34
17	PLA	6.32	5.82		12	PLA	6.02	5.52
24	PLA	5.18	4.80		20	PLA	7.49	5.97
29	PLA	6.61	5.80		27	PLA	6.66	5.53
35	PLA	5.85	5.05		28	PLA	9.01	7.28
40	PLA	5.91	6.22		42	PLA	6.40	5.38
44	PLA	5.67	6.23		44	PLA	7.13	6.27
48	PLA	9.48	8.66		45	PLA	7.14	5.62
22	WP	7.44	5.04		13	PLA	6.98	5.51
25	WP	5.91	5.53		20	PLA	5.37	4.55
31	WP	7.26	5.59		6	PLA	5.26	5.07
33	WP	7.18	5.25		7	PLA	6.80	5.16
34	WP	4.56	4.34		13	WP	5.85	5.37
45	WP	4.72	5.51		19	WP	7.74	6.52
46	WP	5.76	5.50		24	WP	6.40	5.52
47	WP	5.06	4.37		29	WP	7.68	6.17
1	LSUS	6.78	6.18		32	WP	7.50	5.82
10	LSUS	5.06	4.78		34	WP	5.28	4.81
18	LSUS	5.28	4.89		46	WP	7.83	6.32
21	LSUS	8.39	7.98		18	WP	6.91	5.51
28	LSUS	4.85	5.45		4	WP	5.68	6.57
36	LSUS	5.15	5.31		19	WP	5.86	3.66
43	LSUS	5.69	5.16		8	WP	5.71	4.25
52	LSUS	6.32	6.27		11	WP	7.76	4.28
9	HSUS	5.67	4.64		3	LSUS	5.88	4.85
16	HSUS	5.18	5.72		10	LSUS	7.57	6.73
27	HSUS	7.21	6.65		14	LSUS	7.18	6.75
30	HSUS	6.75	6.96		16	LSUS	6.16	5.28
32	HSUS	5.91	5.65		17	LSUS	8.04	6.52
38	HSUS	6.04	5.71		30	LSUS	6.13	5.23
39	HSUS	6.21	7.05		39	LSUS	6.36	5.32
41	HSUS	4.99	5.07		41	LSUS	7.02	6.42
					8	HSUS	8.30	6.63
					9	HSUS	5.28	5.96
					15	HSUS	7.43	6.38
					23	HSUS	8.54	6.40
					25	HSUS	7.63	6.57
					31	HSUS	6.38	6.10
					33	HSUS	6.32	6.66
					40	HSUS	5.83	6.23
					3	HSUS	5.69	6.05
					14	HSUS	5.24	5.62
					5	HSUS	6.56	5.60
					12	HSUS	5.22	5.37

Study 1	Plasma insulin (pM)							
20 min					40 min			
Rat #	Group	0 min	20 min		Rat #	Group	0 min	40 min
2	SED	229.34	322.66		11	SED	469.01	475.90
23	SED	361.23	877.75		21	SED	251.03	261.71
26	SED	344.01	775.14		22	SED	483.13	427.34
37	SED	360.88	537.88		26	SED	349.52	264.46
42	SED	128.79	218.32		35	SED	598.48	361.57
50	SED	185.26	257.58		38	SED	311.98	395.66
15	PLA	683.20	625.69		4	PLA	185.61	342.63
17	PLA	305.79	259.99		12	PLA	129.82	119.49
24	PLA	204.89	258.61		20	PLA	329.20	603.99
29	PLA	230.03	404.96		27	PLA	136.71	177.00
35	PLA	229.68	253.44		28	PLA	466.94	449.72
40	PLA	374.66	625.69		42	PLA	199.72	278.24
44	PLA	164.26	269.28		44	PLA	466.25	391.18
48	PLA	259.30	460.74		45	PLA	245.52	240.70
22	WP	663.22	595.39		13	PLA	271.35	342.98
25	WP	505.85	527.20		20	PLA	270.66	356.06
31	WP	365.01	669.77		6	PLA	169.08	269.28
33	WP	196.28	205.23		7	PLA	213.50	623.62
34	WP	166.67	141.53		13	WP	315.08	207.99
45	WP	196.63	322.31		19	WP	403.93	346.42
46	WP	246.56	558.20		24	WP	272.38	282.71
47	WP	162.88	329.89		29	WP	210.06	130.51
1	LSUS	142.22	143.25		32	WP	429.06	342.29
10	LSUS	376.72	237.95		34	WP	82.99	131.20
18	LSUS	303.72	158.40		46	WP	180.44	171.49
21	LSUS	424.59	326.79		18	WP	307.85	300.96
28	LSUS	126.03	140.15		4	WP	165.29	127.75
36	LSUS	262.40	187.33		19	WP	199.04	178.37
43	LSUS	246.56	200.07		8	WP	194.90	412.19
52	LSUS	163.91	367.08		11	WP	606.75	81.27
9	HSUS	190.08	326.79		3	LSUS	92.98	140.15
16	HSUS	347.45	625.69		10	LSUS	371.21	194.90
27	HSUS	250.34	315.77		14	LSUS	286.16	336.09
30	HSUS	206.27	407.37		16	LSUS	245.87	192.49
32	HSUS	359.85	324.38		17	LSUS	192.84	128.10
38	HSUS	305.79	261.36		30	LSUS	357.09	219.70
39	HSUS	388.43	322.31		39	LSUS	298.55	182.16
41	HSUS	140.15	268.60		41	LSUS	302.00	330.92
					8	HSUS	453.17	325.07
					9	HSUS	275.48	350.90
					15	HSUS	270.66	325.41
					23	HSUS	809.23	348.83
					25	HSUS	298.21	280.30
					31	HSUS	118.11	194.56
					33	HSUS	287.53	265.15
					40	HSUS	57.51	205.23
					3	HSUS	131.20	78.86
					14	HSUS	326.79	430.10
					5	HSUS	158.06	235.54
					12	HSUS	154.96	342.98

Study 1	Plasma growth hormone (ng/ml)						
20 min				40 min			
Rat #	Group	0 min	20 min	Rat #	Group	0 min	40 min
2	SED	2.96	3.61	11	SED	7.42	2.51
23	SED	1.62	1.14	21	SED	16.86	1.86
26	SED	38.70	6.18	22	SED	10.85	2.33
37	SED	14.99	29.29	26	SED	3.07	1.10
42	SED	28.88	16.47	35	SED	40.10	21.04
50	SED	1.82	1.03	38	SED	10.67	1.54
15	PLA	1.41	0.82	4	PLA	1.71	1.17
17	PLA	8.89	3.52	12	PLA	1.11	2.84
24	PLA	2.07	1.01	20	PLA	4.84	2.13
29	PLA	9.03	4.48	27	PLA	1.78	17.45
35	PLA	0.63	24.70	28	PLA	0.66	17.26
40	PLA	1.88	1.46	42	PLA	8.18	3.19
44	PLA	6.12	35.31	44	PLA	0.67	9.25
48	PLA	3.37	1.90	45	PLA	0.63	50.37
22	WP	7.80	3.43	13	PLA	0.88	0.8
25	WP	2.48	1.39	20	PLA	3.31	1.19
31	WP	4.08	2.15	6	PLA	8.08	2.64
33	WP	0.59	73.72	7	PLA	0.84	0.4
34	WP	5.91	19.10	13	WP	2.45	1.05
45	WP	0.94	0.49	19	WP	0.73	9.85
46	WP	0.56	1.22	24	WP	2.93	1.41
47	WP	2.88	1.65	29	WP	4.42	1.96
1	LSUS	1.06	1.44	32	WP	5.29	1.62
10	LSUS	0.55	6.78	34	WP	1.90	2.40
18	LSUS	0.19	1.10	46	WP	1.19	1.17
21	LSUS	0.29	1.20	18	WP	4.21	1.29
28	LSUS	6.42	3.07	4	WP	2.96	0.79
36	LSUS	1.15	0.80	19	WP	0.71	6.89
43	LSUS	0.44	1.07	8	WP	1.92	0.96
52	LSUS	16.46	6.77	11	WP	0.77	87.12
9	HSUS	1.18	0.78	3	LSUS	1.90	1.36
16	HSUS	3.52	1.53	10	LSUS	1.63	1.11
27	HSUS	3.57	2.98	14	LSUS	2.08	3.21
30	HSUS	0.91	32.93	16	LSUS	0.72	13.37
32	HSUS	7.08	0.88	17	LSUS	1.95	1.22
38	HSUS	1.39	0.75	30	LSUS	0.93	0.82
39	HSUS	3.11	1.90	39	LSUS	1.86	1.54
41	HSUS	39.21	36.22	41	LSUS	2.29	1.19
				8	HSUS	0.90	42.55
				9	HSUS	1.22	4.35
				15	HSUS	0.82	66.20
				23	HSUS	3.80	1.42
				25	HSUS	0.87	12.00
				31	HSUS	2.25	1.03
				33	HSUS	1.43	47.63
				40	HSUS	14.56	4.22
				3	HSUS	0.69	0.38
				14	HSUS	1.23	5.71
				5	HSUS	2.35	1.24
				12	HSUS	0.56	49.51

Study 1	Plasma IGF-1 (ng/ml)							
20 min					40 min			
Rat #	Group	0 min	20 min		Rat #	Group	0 min	40 min
2	SED	740.01	772.34		11	SED	969.03	957.41
23	SED	959.78	1085.56		21	SED	1301.33	1432.04
26	SED	902.36	916.32		22	SED	980.71	1032.36
37	SED	1291.33	1351.13		26	SED	1355.89	1077.36
42	SED	934.88	788.58		35	SED	999.47	1087.73
50	SED	1265.14	836.17		38	SED	1162.68	976.77
15	PLA	852.25	832.93		4	PLA	986.57	895.06
17	PLA	952.64	951.63		12	PLA	1047.64	957.94
24	PLA	1156.47	1249.84		20	PLA	939.05	933.31
29	PLA	1219.12	1114.78		27	PLA	782.61	973.00
35	PLA	852.29	838.26		28	PLA	1180.72	1467.31
40	PLA	981.51	1123.33		42	PLA	964.40	1084.40
44	PLA	1052.91	852.29		44	PLA	1191.00	1099.38
48	PLA	1101.20	941.48		45	PLA	1115.54	1099.94
22	WP	910.33	982.34		13	PLA	801.20	799.51
25	WP	1033.39	1165.33		20	PLA	1317.44	1435.64
31	WP	986.46	958.76		6	PLA	708.36	937.08
33	WP	1575.59	1530.21		7	PLA	1143.54	1180.29
34	WP	931.95	896.41		13	WP	1010.17	826.51
45	WP	1008.60	987.50		19	WP	1384.15	1123.18
46	WP	1013.90	1277.72		24	WP	1113.86	1071.68
47	WP	1076.16	1297.96		29	WP	994.60	986.48
1	LSUS	947.56	976.16		32	WP	1116.09	1307.35
10	LSUS	895.41	918.32		34	WP	1130.07	1179.65
18	LSUS	1102.83	1204.44		46	WP	946.90	1014.48
21	LSUS	1247.56	1408.51		18	WP	1411.39	1603.68
28	LSUS	965.86	909.40		4	WP	1006.30	935.61
36	LSUS	839.66	1024.55		19	WP	827.70	921.01
43	LSUS	917.37	882.07		8	WP	1060.08	1559.28
52	LSUS	1349.18	1582.35		11	WP	1146.62	1280.24
9	HSUS	743.68	794.84		3	LSUS	1063.00	988.18
16	HSUS	933.40	1154.25		10	LSUS	1119.80	1354.05
27	HSUS	1182.02	1207.82		14	LSUS	1050.92	1053.66
30	HSUS	1465.72	1528.96		16	LSUS	1057.91	964.40
32	HSUS	974.04	779.07		17	LSUS	1084.01	964.80
38	HSUS	857.22	742.17		30	LSUS	1013.59	1071.68
39	HSUS	1057.54	1065.28		39	LSUS	1208.66	1171.73
41	HSUS	1124.91	1248.46		41	LSUS	1140.17	1189.87
					8	HSUS	890.46	897.11
					9	HSUS	963.22	822.05
					15	HSUS	933.83	970.09
					23	HSUS	1491.47	1335.77
					25	HSUS	957.42	1055.71
					31	HSUS	1099.94	1116.09
					33	HSUS	1004.90	1067.82
					40	HSUS	1142.97	1419.03
					3	HSUS	978.80	1136.05
					14	HSUS	922.03	1186.78
					5	HSUS	1086.32	1161.70
					12	HSUS	1096.15	1175.43

Study 1	Western blot results at 20 min (% of standard)								
Rat #	**Group**	**p-FOXO3A**	**p-AMPK**	**p-NFkB p65**	**p-Akt**	**p-mTOR**	**p-p70S6k**	**p-rpS6**	**α-tubulin**
2	SED	40.10	81.80	17.60	79.00	88.80	5.00	19.20	214.40
23	SED	222.10	64.50	48.00	65.40	100.10	8.20	60.30	247.10
26	SED	155.20	70.20	26.90	66.30	56.70	5.70	39.40	282.70
37	SED	223.40	43.30	27.40	37.20	59.60	0.60	36.10	338.20
42	SED	197.10	112.10	53.30	45.00	98.90	7.70	53.60	242.70
50	SED	241.90	31.10	28.70	26.20	55.90	1.60	24.50	402.60
15	PLA	338.80	32.80	56.90	56.50	167.90	8.20	92.60	337.60
17	PLA	320.10	35.50	57.90	27.00	41.70	3.60	73.80	318.90
24	PLA	240.90	26.00	23.60	14.20	53.50	3.60	70.00	211.10
29	PLA	219.70	42.40	45.90	28.10	90.50	9.60	75.10	318.90
35	PLA	243.30	43.40	23.40	32.10	93.70	3.60	67.40	292.00
40	PLA	182.30	107.20	25.30	26.00	62.40	12.70	72.50	352.60
44	PLA	279.10	60.30	28.30	30.10	79.20	10.90	62.40	249.30
48	PLA	127.50	29.00	60.60	22.60	52.70	5.50	49.80	214.30
22	WP	328.70	27.90	37.30	32.60	206.60	23.20	85.70	309.60
25	WP	264.10	58.80	59.40	26.80	106.70	38.50	79.90	317.50
31	WP	203.70	91.50	59.00	42.70	131.40	45.00	76.30	308.00
33	WP	229.90	27.10	40.30	13.10	105.20	12.40	87.60	225.80
34	WP	422.60	27.00	42.70	16.50	94.40	10.10	97.30	352.80
45	WP	293.70	26.30	43.20	33.00	144.60	23.70	116.50	383.50
46	WP	193.20	201.50	31.00	27.40	113.50	20.60	86.00	211.20
47	WP	332.40	24.80	30.00	17.50	137.70	10.30	78.70	289.20
1	LSUS	377.60	1.70	26.50	8.20	104.50	5.20	76.00	359.10
10	LSUS	365.30	17.00	27.10	24.20	75.80	2.10	52.40	282.70
18	LSUS	213.20	17.40	40.20	22.30	56.70	2.80	42.70	347.20
21	LSUS	279.10	14.70	18.20	18.10	39.60	3.40	42.70	246.50
28	LSUS	407.60	9.50	26.90	20.40	118.50	3.10	46.60	384.80
36	LSUS	323.60	8.70	18.10	13.10	69.40	4.10	62.10	353.60
43	LSUS	260.30	17.50	13.50	14.40	96.80	3.70	74.30	300.20
52	LSUS	277.60	3.30	14.30	24.60	41.60	1.90	49.90	278.50
9	HSUS	332.50	4.40	23.80	14.90	62.90	9.90	73.10	300.80
16	HSUS	376.90	10.70	24.40	21.20	134.00	2.00	78.50	412.10
27	HSUS	204.10	16.20	23.90	25.20	104.00	2.20	60.70	253.80
30	HSUS	376.70	11.90	23.80	21.40	95.50	4.10	52.00	391.10
32	HSUS	318.60	21.60	16.40	18.00	112.40	3.80	94.50	326.40
38	HSUS	252.20	9.20	11.30	38.90	90.50	5.20	76.80	304.90
39	HSUS	275.70	8.70	12.00	21.10	68.30	5.30	78.90	241.80
41	HSUS	229.30	8.40	14.40	19.20	65.70	5.10	63.80	202.60

Study 1	Western blot results at 40 min (% of standard)								
Rat #	**Group**	**p-FOXO3A**	**p-AMPK**	**p-NFkB p65**	**p-Akt**	**p-mTOR**	**p-p70S6k**	**p-rpS6**	**α-tubulin**
11	SED	304.29	300.55	122.32	21.60	64.90	30.57	66.16	178.81
21	SED	207.63	161.25	91.31	45.34	28.14	25.66	57.39	243.99
22	SED	249.84	277.66	124.00	42.03	62.73	78.53	67.30	199.24
26	SED	261.52	257.26	50.04	41.56	50.55	8.43	22.64	237.02
35	SED	314.42	232.92	91.40	66.41	27.95	9.69	38.00	244.13
38	SED	193.72	272.12	99.91	37.01	65.72	22.15	32.81	204.52
4	PLA	302.31	165.78	172.01	30.09	131.09	86.70	80.88	263.86
12	PLA	239.74	91.10	96.41	27.33	87.26	36.29	89.00	196.56
20	PLA	296.98	258.79	108.81	29.71	64.61	57.88	94.43	224.45
27	PLA	344.71	228.96	108.38	54.72	84.43	76.74	61.01	267.76
28	PLA	310.28	198.31	69.88	28.63	48.60	45.92	92.80	232.05
42	PLA	289.35	148.89	101.61	25.87	114.20	59.23	85.18	189.00
44	PLA	202.69	508.40	55.14	31.17	70.19	23.68	53.89	166.73
45	PLA	238.92	201.12	177.19	58.14	78.00	30.70	84.61	190.01
13	PLA	332.40	195.70	142.10	28.70	89.80	54.00	111.70	217.00
20	PLA	290.70	159.00	122.40	34.60	117.60	19.00	71.70	256.90
6	PLA	278.80	262.90	152.70	16.80	183.40	34.20	98.90	232.50
7	PLA	299.40	264.60	100.20	21.30	127.60	28.30	72.40	220.40
13	WP	313.31	164.50	109.07	24.69	83.45	54.00	69.74	213.89
19	WP	303.74	186.12	100.11	22.11	67.48	55.70	87.59	207.91
24	WP	225.55	193.66	116.18	15.05	54.60	89.40	78.67	197.70
29	WP	279.62	247.84	90.41	34.69	105.76	114.03	83.78	263.87
32	WP	352.07	261.02	195.55	46.84	76.39	178.04	86.06	217.12
34	WP	228.17	160.97	100.53	13.92	89.30	59.50	74.42	163.96
46	WP	227.22	189.80	81.76	33.04	128.87	90.42	78.34	194.99
18	WP	370.30	170.10	135.30	18.50	171.00	53.70	113.90	249.50
4	WP	519.30	197.00	164.60	19.30	117.60	64.60	117.60	325.90
19	WP	319.90	245.00	106.00	20.90	152.90	137.10	120.30	237.90
8	WP	442.20	191.70	99.60	27.80	204.80	95.30	116.00	218.00
11	WP	215.57	283.10	76.79	37.09	189.36	87.11	68.79	165.17
3	LSUS	294.53	128.79	125.09	25.49	67.07	79.60	75.85	222.92
10	LSUS	198.60	300.66	89.48	23.15	72.21	54.06	80.63	151.25
14	LSUS	466.80	583.55	87.75	27.27	30.80	39.60	63.37	287.29
16	LSUS	340.07	105.00	77.64	18.26	30.67	70.64	94.64	154.37
17	LSUS	258.95	115.39	123.55	28.72	55.96	88.20	82.94	174.87
30	LSUS	230.06	301.24	86.49	23.48	67.84	56.11	89.37	176.37

39	LSUS	201.11	147.20	74.72	25.88	83.84	88.72	77.24	170.60
41	LSUS	245.89	194.42	235.73	44.43	75.18	38.50	84.16	249.12
8	HSUS	297.29	162.80	155.84	38.21	54.84	88.89	82.39	297.00
9	HSUS	241.91	137.25	88.23	25.97	52.85	35.40	73.98	234.87
15	HSUS	369.40	274.18	103.37	26.22	89.98	123.01	59.17	242.50
23	HSUS	341.32	195.17	140.04	35.70	53.02	76.88	78.86	204.75
25	HSUS	298.80	198.56	141.10	42.76	129.27	97.52	83.74	212.84
31	HSUS	251.25	278.23	109.93	28.80	109.14	72.03	97.92	182.33
33	HSUS	209.11	374.90	76.90	23.13	89.61	67.33	83.74	203.64
40	HSUS	165.73	171.85	110.80	63.10	71.40	11.30	61.47	170.74
3	HSUS	320.10	166.90	136.10	17.60	160.80	44.90	110.50	297.90
14	HSUS	455.70	264.60	129.20	27.70	193.20	56.50	101.20	205.00
5	HSUS	381.50	297.70	147.80	33.20	155.90	54.20	75.20	277.10
12	HSUS	271.20	236.20	103.20	18.60	83.60	21.20	61.00	194.60

APPENDIX I: RAW DATA FOR STUDY 2

Study 2	Plasma glucose (mM)							
1 h					2 h			
Rat #	Group	0 h	1 h		Rat #	Group	0 h	2 h
1	SED	5.83	6.10		17	SED	6.46	6.87
8	SED	5.06	5.23		19	SED	5.53	6.41
12	SED	6.79	5.67		47	SED	5.14	5.01
52	SED	4.93	5.40		51	SED	4.43	5.35
57	SED	5.72	5.83		55	SED	4.44	5.41
73	SED	5.34	5.56		60	SED	5.05	4.08
74	SED	5.71	5.93		69	SED	4.69	4.75
2	PLA	7.06	5.79		24	PLA	5.01	4.78
7	PLA	6.37	6.35		32	PLA	5.12	5.03
34	PLA	6.72	6.05		41	PLA	6.44	5.43
37	PLA	5.94	5.92		66	PLA	5.64	4.85
50	PLA	5.38	4.46		67	PLA	5.79	4.75
54	PLA	4.85	4.77		70	PLA	4.99	3.19
68	PLA	6.11	6.05		71	PLA	5.48	4.96
30	WP	5.38	5.11		21	WP	6.58	5.03
43	WP	5.37	3.97		28	WP	6.35	4.64
46	WP	5.03	5.12		44	WP	5.22	4.27
49	WP	5.81	5.27		45	WP	4.48	3.76
53	WP	7.04	5.96		61	WP	5.06	3.04
56	WP	5.68	5.04		63	WP	6.27	4.06
64	WP	6.64	5.31		65	WP	5.36	4.72
3	CP	6.24	5.12		13	CP	4.34	5.74
4	CP	6.35	5.72		15	CP	6.72	6.53
33	CP	7.24	6.52		29	CP	6.11	6.25
38	CP	5.37	5.55		35	CP	5.07	6.36
42	CP	5.30	5.95		48	CP	6.63	4.43
59	CP	5.20	7.03		58	CP	5.79	5.39
62	CP	6.60	6.42		72	CP	5.31	5.12

Study 2	Plasma insulin (pM)							
1 h					2 h			
Rat #	Group	0 h	1 h		Rat #	Group	0 h	2 h
1	SED	266.53	381.54		17	SED	347.11	384.30
8	SED	131.89	298.90		19	SED	240.01	297.86
12	SED	172.66	274.62		47	SED	179.75	280.99
52	SED	326.10	357.44		51	SED	264.12	157.02
57	SED	174.24	200.76		55	SED	134.30	139.81
73	SED	184.92	403.58		60	SED	281.34	328.17
74	SED	355.72	375.00		69	SED	146.01	276.86
2	PLA	139.19	148.07		24	PLA	165.29	312.67
7	PLA	182.51	122.59		32	PLA	201.10	173.90
34	PLA	159.78	327.82		41	PLA	291.32	203.51
37	PLA	261.71	268.60		66	PLA	231.40	266.18
50	PLA	166.67	263.09		67	PLA	241.74	480.72
54	PLA	173.55	156.34		70	PLA	152.89	160.12
68	PLA	215.56	295.45		71	PLA	210.74	191.46
30	WP	279.61	297.52		21	WP	316.80	289.94
43	WP	190.77	121.90		28	WP	298.90	322.18
46	WP	225.21	297.52		44	WP	376.03	292.01
49	WP	355.37	181.82		45	WP	190.77	139.81
53	WP	210.74	269.28		61	WP	217.63	347.11
56	WP	141.18	180.44		63	WP	471.07	252.07
64	WP	270.66	365.01		65	WP	303.03	322.18
3	CP	154.96	389.46		13	CP	128.44	336.78
4	CP	197.31	239.67		15	CP	247.93	456.61
33	CP	914.26	576.45		29	CP	217.63	128.10
38	CP	146.01	250.00		35	CP	139.81	328.51
42	CP	232.78	469.70		48	CP	303.37	326.45
59	CP	164.60	618.46		58	CP	305.79	385.67
62	CP	362.26	417.36		72	CP	203.17	282.37

Study 2	Plasma growth hormone (ng/ml)						
1 h				2 h			
Rat #	Group	0 h	1 h	Rat #	Group	0 h	2 h
1	SED	2.21	7.63	17	SED	0.54	3.92
8	SED	27.51	2.57	19	SED	0.54	5.74
12	SED	0.92	19.88	47	SED	31.86	1.74
52	SED	5.40	1.62	51	SED	2.16	1.84
57	SED	0.74	11.32	55	SED	22.64	5.09
73	SED	13.11	1.99	60	SED	5.13	6.47
74	SED	5.20	0.79	69	SED	16.56	2.44
2	PLA	0.82	1.66	24	PLA	1.95	4.13
7	PLA	0.43	3.13	32	PLA	0.51	4.92
34	PLA	0.57	6.18	41	PLA	1.11	2.58
37	PLA	2.42	3.06	66	PLA	0.06	12.28
50	PLA	0.03	1.43	67	PLA	0.05	6.87
54	PLA	0.05	10.00	70	PLA	1.47	20.43
68	PLA	0.02	1.37	71	PLA	1.68	1.30
30	WP	0.56	1.58	21	WP	1.30	2.43
43	WP	0.53	1.63	28	WP	7.94	29.63
46	WP	0.99	55.48	44	WP	1.29	3.33
49	WP	0.78	0.80	45	WP	1.98	8.95
53	WP	0.89	1.77	61	WP	2.18	1.40
56	WP	0.86	3.42	63	WP	1.07	1.43
64	WP	0.02	1.38	65	WP	0.05	2.72
3	CP	3.82	10.33	13	CP	0.73	1.21
4	CP	6.44	6.40	15	CP	0.70	4.11
33	CP	0.54	0.21	29	CP	0.80	0.31
38	CP	5.57	42.65	35	CP	1.24	0.58
42	CP	0.27	7.16	48	CP	0.02	6.48
59	CP	1.87	0.94	58	CP	0.84	1.45
62	CP	2.77	0.73	72	CP	0.03	1.55

Study 2	Plasma IGF-1 (ng/ml)							
1 h					2 h			
Rat #	Group	0 h	1 h		Rat #	Group	0 h	2 h
1	SED	1024.36	796.63		17	SED	871.01	649.25
8	SED	809.64	983.56		19	SED	840.06	761.12
12	SED	885.67	965.66		47	SED	940.99	931.29
52	SED	841.71	668.32		51	SED	629.09	492.43
57	SED	643.96	678.90		55	SED	669.50	723.41
73	SED	809.34	717.13		60	SED	740.77	709.65
74	SED	875.72	953.82		69	SED	840.64	796.72
2	PLA	1117.91	725.44		24	PLA	1184.06	985.04
7	PLA	775.18	861.67		32	PLA	987.98	744.62
34	PLA	811.69	739.46		41	PLA	925.93	772.87
37	PLA	661.32	482.33		66	PLA	705.07	889.95
50	PLA	887.99	736.62		67	PLA	645.11	641.66
54	PLA	668.32	536.95		70	PLA	746.01	651.75
68	PLA	956.77	760.53		71	PLA	735.54	721.24
30	WP	839.25	720.68		21	WP	782.73	712.14
43	WP	761.12	651.97		28	WP	829.86	850.31
46	WP	921.62	800.79		44	WP	828.67	927.00
49	WP	735.39	545.43		45	WP	614.38	588.76
53	WP	599.84	569.07		61	WP	677.72	695.50
56	WP	757.59	797.84		63	WP	794.02	727.72
64	WP	802.15	656.70		65	WP	708.37	602.09
3	CP	717.83	618.84		13	CP	1004.61	843.42
4	CP	1053.12	819.72		15	CP	931.29	912.93
33	CP	1004.61	843.42		29	CP	649.29	644.37
38	CP	688.36	519.14		35	CP	538.01	446.79
42	CP	642.81	518.10		48	CP	729.28	615.51
59	CP	758.84	594.29		58	CP	735.54	667.88
62	CP	1097.30	819.92		72	CP	779.20	769.84

Study 2	Plasma corticosterone (ng/ml)							
1 h					2 h			
Rat #	Group	0 h	1 h		Rat #	Group	0 h	2 h
1	SED	174.83	187.82		17	SED	130.32	99.97
8	SED	50.39	119.19		19	SED	150.02	93.30
12	SED	71.42	161.57		47	SED	138.26	102.75
52	SED	198.89	162.78		51	SED	243.02	99.00
57	SED	135.79	159.92		55	SED	131.62	83.78
73	SED	70.03	96.38		60	SED	158.68	218.38
74	SED	212.10	213.68		69	SED	162.34	124.25
2	PLA	239.63	173.58		24	PLA	236.22	120.44
7	PLA	248.49	167.68		32	PLA	187.43	63.43
34	PLA	229.01	136.22		41	PLA	349.60	131.26
37	PLA	181.14	111.76		66	PLA	216.68	112.63
50	PLA	322.37	192.79		67	PLA	362.32	195.82
54	PLA	322.37	203.59		70	PLA	324.89	130.60
68	PLA	231.87	201.35		71	PLA	341.17	137.86
30	WP	307.94	210.63		21	WP	294.98	128.47
43	WP	167.68	153.40		28	WP	190.23	93.99
46	WP	140.32	49.65		44	WP	246.65	63.90
49	WP	382.63	200.45		45	WP	189.81	55.87
53	WP	348.48	243.54		61	WP	218.38	227.05
56	WP	289.06	194.30		63	WP	164.76	104.74
64	WP	184.18	81.97		65	WP	244.24	138.89
3	CP	154.78	183.82		13	CP	138.26	53.47
4	CP	194.51	172.74		15	CP	118.31	50.77
33	CP	239.44	198.89		29	CP	206.41	139.29
38	CP	234.24	134.73		35	CP	307.65	174.22
42	CP	351.21	255.19		48	CP	464.89	163.70
59	CP	121.51	97.24		58	CP	271.00	99.43
62	CP	202.85	46.96		72	CP	181.47	152.97

Study 2		Western blot results at 1 h		
Rat #	Group	Puromycin labeled peptide	Free puromycin	Protein synthesis
		% of standard	(pmol/mg protein)	(PLP/free puromycin)
1	SED	131.00	4.40	29.76
8	SED	100.00	6.02	16.61
12	SED	113.60	2.27	50.01
52	SED	207.10	14.44	14.35
57	SED	229.90	5.12	44.89
73	SED	106.80	7.47	14.30
74	SED	178.40	7.10	25.13
77	SED	138.20	7.91	17.47
82	SED	110.50	8.55	12.92
88	SED	174.30	7.70	22.64
2	PLA	58.50	7.44	7.86
7	PLA	77.40	7.35	10.53
34	PLA	70.00	2.85	24.52
37	PLA	101.00	4.10	24.64
50	PLA	165.00	6.21	26.58
54	PLA	67.20	8.19	8.21
68	PLA	166.20	6.94	23.95
78	PLA	76.10	8.36	9.10
91	PLA	87.80	9.86	8.90
97	PLA	72.50	8.14	8.91
30	WP	208.10	10.50	19.82
43	WP	87.40	6.48	13.49
46	WP	183.10	10.87	16.84
49	WP	134.72	7.16	18.82
53	WP	134.80	7.42	18.18
56	WP	209.10	4.92	42.52
64	WP	83.30	3.74	22.25
80	WP	99.10	6.94	14.28
93	WP	80.80	6.37	12.68
94	WP	126.80	7.23	17.54
3	CP	177.20	3.36	52.74
4	CP	118.10	6.89	17.14
33	CP	203.10	6.64	30.59
38	CP	307.10	9.36	32.80
42	CP	172.00	8.32	20.66
59	CP	108.00	6.18	17.48
62	CP	204.90	4.24	48.28
79	CP	81.60	6.12	13.33
90	CP	145.20	4.06	35.76
96	CP	157.50	6.61	23.83

Study 2		Western blot results at 1 h			
Rat #	Group	p-4E-BP1 polyclonol	p-mTOR	p-p70S6k	p-rpS6
		(% of γ isoform)	(% of standard)		
1	SED	33.60	32.00	112.35	80.50
8	SED	41.10	48.50	155.56	47.10
12	SED	37.70	53.70	172.84	40.90
52	SED	39.50	34.90	86.42	72.70
57	SED	34.10	13.50	86.42	22.50
73	SED	27.20	13.50	51.85	24.40
74	SED	41.60	30.70	34.57	47.60
77	SED	33.56	52.92	70.46	102.62
82	SED	32.43	26.60	81.25	99.18
88	SED	34.90	32.00	148.29	151.96
2	PLA	14.90	63.40	185.80	99.30
7	PLA	25.80	50.60	341.36	106.40
34	PLA	26.00	43.10	146.91	97.00
37	PLA	39.40	32.70	151.23	88.60
50	PLA	22.60	51.00	272.22	175.70
54	PLA	18.20	24.20	237.65	91.90
68	PLA	41.00	18.90	207.41	93.80
78	PLA	21.34	37.69	126.54	121.66
91	PLA	20.29	41.40	167.99	141.50
97	PLA	21.90	102.70	193.53	177.35
30	WP	48.60	74.20	639.51	167.90
43	WP	32.20	38.70	142.59	49.00
46	WP	35.80	35.90	103.70	73.30
49	WP	34.53	52,44	245.68	114.38
53	WP	40.20	39.30	341.36	89.10
56	WP	34.50	53.20	90.74	85.60
64	WP	31.80	34.10	129.63	68.80
80	WP	32.38	74.55	169.16	110.06
93	WP	29.95	81.50	304.26	180.29
94	WP	25.34	40.54	290.16	205.39
3	CP	35.20	96.10	216.05	107.40
4	CP	29.50	70.20	371.60	211.90
33	CP	43.20	63.90	639.51	143.60
38	CP	42.30	64.00	164.20	85.50
42	CP	42.30	47.30	220.37	89.40
59	CP	31.60	35.70	362.96	71.40
62	CP	42.60	55.70	259.26	135.80
79	CP	32.07	125.10	368.37	189.14
90	CP	27.02	116.80	192.41	159.27
96	CP	20.30	30.72	190.19	164.13

Study 2		Western blot results at 1 h						
Rat #	Group	p-Akt	p-GSKβ	p-GSKα	p-eIF2Bε	p-AMPK	p-FOXO3A	α-tubulin
		(% of standard)						
1	SED	22.80	57.40	47.40	156.50	80.30	137.30	273.32
8	SED	13.60	23.80	32.20	107.00	86.10	102.60	253.30
12	SED	15.10	53.60	69.70	85.70	43.70	126.70	198.83
52	SED	22.40	75.20	51.90	111.60	49.10	75.90	188.03
57	SED	16.90	48.60	56.40	85.80	90.10	73.10	107.38
73	SED	12.10	65.10	60.80	105.30	68.20	128.10	209.32
74	SED	13.00	40.20	44.80	110.50	54.10	94.90	198.00
77	SED	49.48	76.81	65.90	119.77	30.20	104.51	189.37
82	SED	34.40	86.44	94.80	109.44	20.34	107.00	170.95
88	SED	25.56	69.19	49.60	127.29	10.40	82.27	219.83
2	PLA	10.20	35.60	31.40	104.50	143.40	144.60	220.74
7	PLA	10.90	33.80	36.00	102.80	58.40	118.90	201.75
34	PLA	18.50	37.00	34.50	94.90	98.90	236.20	198.43
37	PLA	20.30	33.70	41.10	124.10	116.40	105.80	199.74
50	PLA	11.60	68.70	67.40	117.40	73.50	169.40	175.47
54	PLA	8.50	67.00	82.00	79.80	134.20	105.90	157.38
68	PLA	16.00	51.30	55.90	93.10	158.60	95.30	198.38
78	PLA	19.75	106.39	83.60	110.00	20.21	155.85	248.43
91	PLA	23.53	127.30	83.80	130.73	90.80	150.31	229.73
97	PLA	16.03	88.74	40.37	134.14	20.90	128.64	187.74
30	WP	24.30	31.10	42.90	182.60	205.10	197.90	199.08
43	WP	10.20	58.50	49.10	96.50	195.40	152.00	179.46
46	WP	4.60	41.40	34.70	88.20	121.30	173.60	166.95
49	WP	18.83	58.33	47.44	104.27	89.23	146.13	204.86
53	WP	14.50	42.80	50.10	70.90	121.00	102.10	222.20
56	WP	15.40	32.00	28.30	76.60	114.20	84.00	183.74
64	WP	13.20	35.00	31.60	66.30	76.90	66.00	176.95
80	WP	36.61	81.76	87.20	110.99	20.67	175.60	190.74
93	WP	31.14	89.19	50.81	116.79	68.80	183.75	201.88
94	WP	19.56	113.19	52.28	129.59	89.70	180.26	119.84
3	CP	12.10	42.20	39.40	76.50	164.20	175.10	198.74
4	CP	9.30	58.50	44.40	133.80	107.30	144.10	200.83
33	CP	30.60	37.80	42.10	123.30	207.10	286.30	248.88
38	CP	13.70	36.40	29.50	102.80	120.80	132.80	228.83
42	CP	14.40	41.50	33.00	114.00	94.70	124.50	202.48
59	CP	20.20	63.50	55.40	72.30	170.30	120.90	169.84
62	CP	10.10	37.40	30.40	83.60	117.60	135.00	164.99
79	CP	23.76	103.22	96.60	148.34	28.19	216.37	200.93
90	CP	31.81	86.11	48.98	103.88	76.00	168.86	269.88
96	CP	22.74	101.90	49.89	123.87	97.40	108.90	177.95

Study 2		Western blot results at 2 h		
Rat #	Group	Puromycin labeled peptide	Free puromycin	Protein synthesis
		% of standard	(pmol/mg protein)	(PLP/free puromycin)
17	SED	144.10	4.94	29.17
19	SED	197.00	2.83	24.30
47	SED	180.00	11.01	16.35
51	SED	268.10	4.24	24.30
55	SED	192.90	6.10	31.60
60	SED	122.00	7.02	17.38
69	SED	288.30	6.73	42.84
75	SED	100.00	6.04	16.56
86	SED	159.75	8.62	18.53
98	SED	215.40	9.81	21.96
24	PLA	109.80	7.89	13.91
32	PLA	148.50	6.16	24.10
41	PLA	124.70	4.86	25.65
66	PLA	222.30	6.71	33.12
67	PLA	152.80	7.01	21.80
70	PLA	107.30	9.49	11.31
71	PLA	265.50	7.07	37.54
76	PLA	135.10	10.68	12.65
84	PLA	166.99	6.07	27.51
89	PLA	159.22	7.33	21.72
21	WP	104.80	4.75	22.05
28	WP	138.90	9.25	15.02
44	WP	162.80	13.93	11.69
45	WP	283.80	6.67	42.52
61	WP	233.30	3.67	63.57
63	WP	130.90	3.05	42.92
65	WP	98.90	8.10	12.21
81	WP	177.40	5.68	31.23
92	WP	116.00	6.55	17.71
95	WP	140.90	7.15	19.71
13	CP	194.90	5.00	38.99
15	CP	137.90	7.06	19.53
29	CP	179.50	9.25	19.41
35	CP	168.80	8.39	20.13
48	CP	270.30	12.83	21.07
58	CP	172.80	2.82	61.28
72	CP	325.40	7.06	46.09
83	CP	112.80	5.94	18.99
85	CP	139.80	6.77	20.65
87	CP	130.90	5.50	23.80

Study 2		Western blot results at 2 h			
Rat #	Group	p-4E-BP1 polyclonol	p-mTOR	p-p70S6k	p-rpS6
		(% of γ isoform)	(% of standard)		
17	SED	30.00	26.40	103.70	26.90
19	SED	49.30	30.70	259.26	78.10
47	SED	42.70	57.30	116.67	85.50
51	SED	41.70	82.30	90.74	45.00
55	SED	24.90	37.30	21.60	27.10
60	SED	17.60	21.40	17.28	19.80
69	SED	31.10	25.50	64.81	16.40
75	SED	25.43	21.74	162.37	90.87
86	SED	22.06	51.35	96.94	19.03
98	SED	37.95	33.64	239.89	111.94
24	PLA	38.70	69.30	816.67	227.50
32	PLA	46.40	60.30	566.05	166.00
41	PLA	28.40	22.70	95.06	42.00
66	PLA	52.30	83.60	466.67	171.70
67	PLA	42.70	22.40	82.10	93.10
70	PLA	18.80	36.90	30.25	34.00
71	PLA	45.20	31.40	112.35	156.00
76	PLA	38.43	45.62	345.69	133.73
84	PLA	32.52	42.64	557.50	139.58
89	PLA	38.16	46.10	341.37	129.29
21	WP	45.60	54.30	639.51	187.20
28	WP	50.70	40.40	916.05	131.00
44	WP	38.40	18.90	406.17	143.30
45	WP	44.20	29.60	272.22	63.60
61	WP	44.90	28.20	211.73	97.40
63	WP	27.20	52.40	259.26	72.00
65	WP	51.50	48.70	211.73	86.20
81	WP	34.08	33.67	259.14	118.40
92	WP	33.24	78.50	443.85	167.89
95	WP	34.59	37.54	304.26	150.96
13	CP	46.50	62.20	1075.93	208.70
15	CP	42.00	16.90	700.00	114.70
29	CP	38.80	75.00	142.59	94.70
35	CP	57.90	82.40	725.93	183.40
48	CP	35.00	48.40	211.73	93.00
58	CP	34.00	33.40	216.05	56.50
72	CP	44.80	82.30	419.14	128.20
83	CP	33.79	54.07	363.78	124.52
85	CP	35.16	98.65	406.40	139.74
87	CP	36.57	69.64	543.52	160.71

Study 2		Western blot results at 2 h						
Rat #	Group	p-Akt	p-GSKβ	p-GSKα	p-eIF2Bε	p-AMPK	p-FOXO3A	α-tubulin
		(% of standard)						
17	SED	16.30	34.30	46.20	101.00	9.40	131.40	198.93
19	SED	13.30	48.10	95.00	126.40	13.20	125.40	191.02
47	SED	15.60	60.30	50.30	145.20	71.10	123.80	220.98
51	SED	19.80	49.60	44.80	135.00	68.60	123.30	158.98
55	SED	17.60	79.70	62.00	181.40	52.20	100.80	198.83
60	SED	14.90	42.20	46.60	96.30	30.20	75.20	224.95
69	SED	16.80	41.60	45.10	89.10	51.40	62.50	290.84
75	SED	26.10	92.29	63.31	112.10	46.10	135.17	180.84
86	SED	17.69	74.47	47.86	109.74	47.20	146.14	199.84
98	SED	35.90	53.97	33.72	94.27	32.60	94.22	111.84
24	PLA	21.50	27.70	26.30	124.70	60.00	181.10	200.84
32	PLA	11.40	30.90	26.60	143.00	34.30	246.00	240.75
41	PLA	12.50	78.00	64.30	96.70	53.90	109.00	289.75
66	PLA	22.50	74.50	56.10	123.10	79.60	100.30	199,74
67	PLA	16.30	55.50	41.80	66.40	105.10	141.30	164.84
70	PLA	9.70	41.20	31.80	105.90	70.50	135.50	119.84
71	PLA	10.70	26.60	41.50	116.60	71.20	99.00	188.65
76	PLA	19.25	77.79	45.74	97.20	65.00	101.21	178.74
84	PLA	29.42	93.04	45.70	94.70	75.50	145.69	240.74
89	PLA	17.03	56.14	42.40	107.59	67.23	139.90	220.74
21	WP	17.00	32.30	37.10	118.30	145.60	135.40	272.74
28	WP	12.20	37.50	54.90	95.30	148.70	185.00	165.84
44	WP	6.70	40.50	36.50	85.60	112.00	135.40	167.47
45	WP	5.10	47.80	34.80	82.90	103.00	161.20	237.70
61	WP	19.70	43.80	39.10	95.20	137.50	202.70	177.85
63	WP	9.80	36.90	25.10	105.00	138.50	76.30	190.47
65	WP	20.10	45.60	46.00	86.00	144.90	87.90	228.77
81	WP	20.12	84.98	46.78	112.03	86.70	136.61	272.64
92	WP	26.57	110.44	59.27	86.96	85.70	183.33	190.74
95	WP	30.06	92.26	49.56	108.51	122.40	179.82	277.74
13	CP	17.30	66.20	93.80	106.20	43.00	122.20	116.64
15	CP	10.50	43.10	77.20	78.00	31.20	144.90	187.64
29	CP	17.10	29.60	17.40	83.10	38.00	145.40	190.74
35	CP	10.90	31.10	27.00	103.70	27.90	232.60	200.57
48	CP	17.40	48.50	56.50	140.20	165.70	106.10	250.75
58	CP	17.70	62.90	64.10	129.90	70.30	110.00	197.76
72	CP	22.70	47.00	44.40	100.10	97.80	125.50	177.00
83	CP	36.16	102.47	54.48	117.10	62.10	162.22	276.64
85	CP	25.17	90.44	51.56	94.46	87.20	186.96	188.84
87	CP	28.80	100.18	56.09	133.20	63.50	170.65	190.74

APPENDIX J: RAW DATA FOR STUDY 3

Study 3		Rat physical characteristics					
Rat #	Group	Food intake	Total calorie	Initial BW	Final BW	FHL muscle	Adipose
		(g)	(kcal)	(g)	(g)	(mg)	(g)
1	SED	28.0	98.9	329	533	704	11.12
2	SED	29.2	103.1	349	563	791	12.00
12	SED	29.0	102.3	416	576	780	10.87
16	SED	28.0	98.8	420	578	817	7.93
31	SED	31.2	110.2	365	554	730	8.56
34	SED	29.5	104.2	344	545	730	9.44
37	SED	28.2	99.6	320	528	650	7.71
3	PLA	27.8	98.0	407	544	881	5.15
10	PLA	27.2	95.9	411	556	880	7.82
13	PLA	28.2	99.7	415	572	860	13.10
24	PLA	28.7	101.4	383	525	950	6.10
27	PLA	27.5	96.9	380	497	830	6.12
28	PLA	26.9	95.0	387	478	780	6.35
36	PLA	26.7	94.3	341	464	780	7.06
38	PLA	28.9	101.9	347	521	850	10.01
4	WH	25.4	90.0	386	503	830	6.79
6	WH	29.1	103.0	406	566	848	10.02
11	WH	27.4	97.0	402	544	885	8.15
14	WH	25.9	91.8	393	523	838	8.79
22	WH	30.0	106.3	392	580	847	11.80
26	WH	30.2	106.9	405	572	820	11.05
32	WH	24.9	88.2	324	434	740	7.15
39	WH	27.8	98.3	344	514	830	7.40
5	CW	25.8	92.2	398	506	840	7.40
7	CW	24.6	87.7	394	486	790	8.70
15	CW	23.9	85.3	380	492	821	7.68
25	CW	28.8	102.8	388	528	838	7.25
30	CW	28.8	102.6	393	511	967	7.54
33	CW	29.5	105.2	374	592	840	8.46
35	CW	29.3	104.2	335	498	852	7.15
40	CW	26.6	95.0	326	502	840	8.47

Study 3		DEXA-week 0			
Rat #	Group	Fat %	Lean mass %	Fat mass (g)	Lean mass (g)
1	SED	35.1	64.9	112	207
2	SED	33.5	66.5	113	223
12	SED	34.5	65.5	127	241
16	SED	35.3	64.7	129	236
31	SED	39	61	125	196
34	SED	37.3	62.7	112	188
37	SED	35.4	64.6	98	179
3	PLA	33.7	66.3	128	252
10	PLA	40	60	148	222
13	PLA	40.2	59.8	141	209
24	PLA	31.5	68.5	112	244
27	PLA	36.2	63.8	129	227
28	PLA	36.1	63.9	129	229
36	PLA	42.2	57.8	120	164
38	PLA	38.5	61.5	109	174
4	WH	36.8	63.2	135	231
6	WH	38.5	61.5	140	225
11	WH	35.4	64.6	124	226
14	WH	34.6	65.4	120	226
22	WH	36.2	63.8	134	236
26	WH	34	66	122	238
32	WH	35.2	64.8	115	211
39	WH	38.4	61.6	108	173
5	CW	36.5	63.5	138	240
7	CW	36.1	63.9	134	238
15	CW	36.3	63.7	123	215
25	CW	38.8	61.2	140	220
30	CW	33.9	66.1	124	242
33	CW	37.6	62.4	118	197
35	CW	36	64	108	192
40	CW	37.9	62.1	104	171

Study 3		DEXA-week 4			
Rat #	Group	Fat %	Lean mass %	Fat mass (g)	Lean mass (g)
1	SED	43.4	56.6	187	244
2	SED	38.7	61.3	172	272
12	SED	42.1	57.9	218	299
16	SED	43.8	56.2	218	280
31	SED	49	51	231	240
34	SED	40.9	59.1	188	271
37	SED	40.7	59.3	176	256
3	PLA	34.6	65.4	167	315
10	PLA	41.4	58.6	204	288
13	PLA	53.5	46.5	265	232
24	PLA	38.4	61.6	172	275
27	PLA	34.9	65.1	152	283
28	PLA	43.7	56.3	183	236
36	PLA	51.3	48.7	210	200
38	PLA	50.9	49.1	222	215
4	WH	39.6	60.4	177	270
6	WH	44.8	55.2	220	271
11	WH	40.8	59.2	194	284
14	WH	44.8	55.2	202	249
22	WH	46.5	53.5	226	259
26	WH	44.7	55.3	215	266
32	WH	42.7	57.3	164	220
39	WH	45.2	54.8	197	239
5	CW	43.9	56.1	199	255
7	CW	41.4	58.6	179	254
15	CW	44.1	55.9	190	240
25	CW	42.2	57.8	187	256
30	CW	40.8	59.2	182	264
33	CW	46.8	53.2	235	267
35	CW	46.8	53.2	201	228
40	CW	45.8	54.2	183	216

Study 3		DEXA-week 8			
Rat #	Group	Fat %	Lean mass %	Fat mass (g)	Lean mass (g)
1	SED	46.4	53.6	234	270
2	SED	43.1	56.9	231	305
12	SED	44.9	55.1	250	307
16	SED	44.8	55.2	247	304
31	SED	48.1	51.9	260	281
34	SED	39.9	60.1	211	319
37	SED	34.5	65.5	176	334
3	PLA	35.3	64.7	180	330
10	PLA	45.7	54.3	245	291
13	PLA	55	45	299	245
24	PLA	39.2	60.8	200	311
27	PLA	36.8	63.2	178	305
28	PLA	41.8	58.2	189	263
36	PLA	47.8	52.2	212	232
38	PLA	47.4	52.6	234	259
4	WH	38.6	61.4	251	294
6	WH	47	53	184	284
11	WH	44.5	55.5	234	292
14	WH	45.6	54.4	233	278
22	WH	45.6	54.4	255	304
26	WH	51.7	48.3	281	263
32	WH	38.4	61.6	157	253
39	WH	44.4	55.6	216	270
5	CW	41.3	58.7	207	287
7	CW	45	55	202	253
15	CW	45.3	54.7	213	258
25	CW	44.5	55.5	222	277
30	CW	42	58	208	287
33	CW	47	53	266	300
35	CW	43.3	56.7	205	269
40	CW	46.3	53.7	226	256

Study 3		Maximal carrying capacity (g)									
Rat #	**Group**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
3	PLA	485	545	605	635	665	725	785	845	905	935
10	PLA	488	578	668	728	788	788	908	968	998	1028
13	PLA	431	491	521	551	581	611	641	671	701	701
24	PLA	467	557	587	677	737	797	797	827	827	827
27	PLA	435	555	615	675	735	765	855	945	1005	1035
28	PLA	501	561	621	681	681	711	771	801	831	921
36	PLA	495	585	675	705	735	765	795	825	855	915
38	PLA	500	530	680	800	830	890	890	920	980	1010
4	WH	469	529	589	619	679	709	769	769	799	859
6	WH	484	514	544	544	604	634	694	724	724	754
11	WH	511	541	601	721	811	871	931	961	1051	1081
14	WH	414	474	474	504	624	714	774	774	834	864
22	WH	474	533	593	653	713	773	803	863	893	953
26	WH	423	513	543	603	633	693	723	723	753	753
32	WH	423	543	633	693	783	843	903	933	963	993
39	WH	498	588	648	708	768	798	858	888	918	978
5	CW	448	478	538	598	658	718	808	868	958	988
7	CW	415	475	505	535	625	685	745	805	835	865
15	CW	435	465	525	585	645	735	765	765	825	855
25	CW	501	591	651	741	831	891	951	981	1041	1071
30	CW	534	624	714	774	834	954	1014	1044	1074	1134
33	CW	430	490	580	640	730	820	880	940	970	1000
35	CW	461	551	581	701	821	881	911	971	1031	1091
40	CW	424	484	544	694	724	784	814	904	934	964

Maximal carrying capacity (g)										% increase	% increase
Rat #	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
3	965	995	1085	1085	1115	1145	1235	1235	1295	1325	273
10	1028	1028	1058	1058	1088	1088	1118	1178	1208	1298	266
13	731	761	791	881	971	971	1001	1031	1061	1091	253
24	887	947	1007	1067	1157	1187	1217	1247	1247	1247	267
27	1065	1125	1155	1215	1245	1275	1275	1335	1365	1395	321
28	981	1011	1071	1141	1201	1231	1291	1291	1291	1291	258
36	945	945	975	1035	1095	1095	1125	1155	1155	1185	239
38	1070	1170	1230	1290	1290	1320	1350	1380	1380	1410	282
4	919	919	949	1009	1039	1099	1159	1159	1189	1219	260
6	784	814	844	874	934	1024	1054	1084	1114	1174	243
11	1141	1171	1231	1261	1321	1351	1351	1411	1441	1471	288
14	954	1014	1044	1104	1164	1194	1224	1254	1284	1314	317
22	1043	1073	1133	1163	1223	1253	1283	1313	1343	1343	283
26	783	813	843	903	933	933	963	963	993	993	235
32	1053	1113	1143	1203	1233	1263	1293	1293	1293	1323	313
39	1008	1068	1128	1188	1218	1218	1218	1248	1248	1248	251
5	1018	1048	1138	1138	1168	1228	1258	1258	1258	1288	288
7	955	985	1015	1075	1105	1135	1135	1165	1165	1195	288
15	915	1005	1035	1095	1155	1215	1245	1245	1275	1275	293
25	1101	1176	1236	1326	1356	1386	1416	1446	1446	1476	295
30	1224	1284	1344	1374	1434	1464	1524	1614	1674	1704	319
33	1060	1120	1180	1210	1270	1330	1360	1360	1390	1390	323
35	1151	1221	1341	1371	1400	1400	1470	1470	1470	1500	325
40	1024	1054	1114	1144	1174	1204	1234	1264	1264	1294	305

Study 3	Muscle morphological results					
Rat #	Group	Fiber CSA	Muscle length	Muscle CSA	# of Nuclei	Myonuclear domain
		(µm2)	(cm)	(mm2)	(per fiber)	(µm2/nucleus)
1	SED	1553.27	3.5	59.9	1.61	952.80
2	SED	1526.78	3.3	71.4	1.56	1021.52
12	SED	1718.67	3.3	70.4	1.35	1220.42
16	SED	2456.83	3.3	73.8	1.47	1530.72
31	SED	2373.10	3.1	70.2	1.40	1890.46
34	SED	2228.79	3.2	68.0	1.24	2076.47
37	SED	2371.95	3.2	60.5	1.17	1978.12
3	PLA	3423.42	3.2	82.0	1.42	2517.34
10	PLA	2736.32	3.1	84.6	1.61	1711.32
13	PLA	3531.20	3.2	80.1	1.87	1806.18
24	PLA	3278.41	3.2	88.5	1.69	1787.10
27	PLA	3034.44	3.1	79.8	1.78	1529.08
28	PLA	1894.70	3.1	75.0	1.21	1709.97
36	PLA	2374.20	3.1	75.0	1.39	1742.25
38	PLA	3317.99	3.1	81.7	1.46	2123.67
4	WH	2408.35	3.4	72.7	1.92	1315.47
6	WH	4217.08	3.1	81.5	1.77	2215.13
11	WH	2260.02	3.3	79.9	1.46	1649.38
14	WH	3234.37	3.2	78.0	1.56	2173.37
22	WH	3612.27	3.2	78.9	1.83	2102.05
26	WH	2817.49	3.3	74.0	1.52	1825.42
32	WH	1728.17	3.2	68.9	1.14	1364.42
39	WH	3563.22	3.1	79.8	1.54	2315.19
5	CW	3819.27	3.1	80.7	1.78	2173.05
7	CW	2735.92	3.1	75.9	1.56	1784.34
15	CW	4104.90	3.2	76.5	1.77	2354.06
25	CW	2362.83	3	78.3	1.32	1626.76
30	CW	2513.45	3.1	93.0	1.51	1863.93
33	CW	2395.04	3.2	78.2	1.38	1790.91
35	CW	2703.99	3.2	79.3	1.48	1729.04
40	CW	3286.53	3.1	80.7	1.36	2284.58

Study 3	Total and myofibrillar proteins				
Rat #	Group	Total protein concentration	Total protein	Myofibrillar concentration	Myofibrillar protein
		(mg/g)	(mg/muscle)	(mg/g)	(mg/muscle)
1	SED	169.87	119.59	73.37	51.65
2	SED	167.29	132.33	78.08	61.76
12	SED	169.08	131.88	61.98	48.34
16	SED	204.03	166.69	84.95	69.40
31	SED	185.75	135.60	92.59	67.59
34	SED	196.74	143.62	74.59	54.45
37	SED	167.35	108.78	80.12	52.08
3	PLA	203.06	178.90	83.84	73.86
10	PLA	170.78	150.29	76.04	66.91
13	PLA	154.83	133.16	71.53	61.52
24	PLA	176.01	167.20	70.63	67.10
27	PLA	211.64	175.66	91.97	76.34
28	PLA	186.05	145.12	96.94	75.61
36	PLA	197.91	154.37	74.46	58.08
38	PLA	181.80	154.53	81.18	69.01
4	WH	170.29	141.34	68.88	57.17
6	WH	176.38	149.57	78.79	66.81
11	WH	195.10	172.66	64.73	57.28
14	WH	195.49	163.82	91.73	76.87
22	WH	162.68	137.79	99.13	83.96
26	WH	193.15	158.38	85.82	70.37
32	WH	153.93	113.91	84.60	62.60
39	WH	211.27	175.36	83.40	69.22
5	CW	164.36	138.06	86.76	72.88
7	CW	203.82	161.02	83.13	65.68
15	CW	171.90	141.13	110.37	90.62
25	CW	182.05	152.56	82.93	69.49
30	CW	256.07	247.62	90.69	87.70
33	CW	185.57	155.04	99.28	83.40
35	CW	174.97	149.07	81.67	69.58
40	CW	187.36	157.38	87.02	73.10

Study 3	Western blot results (% of standard)						
Rat #	**Group**	**IGF-1**	**Myostatin**	**Pax 7**	**Myo D**	**Myogenin**	**α-tubulin**
1	SED	91.40	36.92	35.88	31.82	34.75	128.50
2	SED	65.00	18.20	34.10	24.50	20.20	137.00
12	SED	89.00	45.20	40.40	58.40	40.40	122.70
16	SED	69.50	91.80	89.10	64.50	77.00	97.20
31	SED	93.40	19.40	17.60	17.70	22.90	134.60
34	SED	105.20	22.70	17.00	9.30	24.80	110.50
37	SED	59.80	24.20	17.10	16.50	23.20	119.10
3	PLA	77.90	46.40	61.00	60.20	47.40	122.70
10	PLA	92.90	29.60	38.40	42.00	37.50	118.10
13	PLA	99.80	39.90	32.20	46.10	35.00	115.80
24	PLA	105.10	34.10	34.70	20.90	41.70	116.80
27	PLA	98.80	23.40	20.60	16.30	40.00	144.70
28	PLA	97.10	34.60	26.40	22.30	40.20	110.60
36	PLA	43.10	27.20	25.30	20.00	24.80	113.70
38	PLA	97.00	30.50	13.10	11.30	39.50	128.80
4	WH	83.20	72.80	104.00	74.20	57.40	131.60
6	WH	95.00	19.70	13.20	16.90	22.70	109.30
11	WH	86.10	57.90	39.50	45.10	41.70	138.20
14	WH	97.90	40.10	42.50	23.80	56.60	123.80
22	WH	92.80	16.80	21.70	15.40	33.90	121.20
26	WH	88.10	32.90	26.50	22.00	39.00	103.00
32	WH	100.30	26.30	17.40	17.00	31.60	135.10
39	WH	100.80	55.10	47.30	40.80	92.10	143.50
5	CW	95.00	56.90	74.30	54.70	41.60	132.60
7	CW	107.70	17.20	21.80	32.60	24.70	100.70
15	CW	92.00	66.40	67.50	55.40	72.30	138.90
25	CW	124.30	20.70	30.00	14.90	28.00	121.20
30	CW	135.20	32.20	32.40	17.70	52.30	141.90
33	CW	80.60	22.10	21.80	20.00	25.20	159.30
35	CW	71.40	48.20	25.90	17.50	59.50	100.40
40	CW	122.00	41.00	20.50	15.50	50.40	100.60

study 3	Plasma corticosterone (ng/ml)			
Rat #	Group	week 0	week 4	week 8
1	SED	88.37	144.87	37.30
2	SED	77.96	65.32	42.59
12	SED	13.29	3.01	39.26
16	SED	53.07	31.51	23.50
31	SED	72.33	22.38	77.20
34	SED	26.36	13.62	121.10
37	SED	4.08	41.32	60.36
3	PLA	40.45	0.64	68.78
10	PLA	23.44	3.88	237.98
13	PLA	2.62	43.65	8.35
24	PLA	10.41	85.82	127.92
27	PLA	32.29	30.01	79.76
28	PLA	14.00	59.54	154.15
36	PLA	55.19	19.62	109.21
38	PLA	26.21	56.34	62.05
4	WH	8.99	1.66	28.39
6	WH	8.92	2.28	82.09
11	WH	33.91	13.07	7.70
14	WH	27.10	33.09	12.86
22	WH	81.00	40.90	63.49
26	WH	18.32	32.46	56.73
32	WH	15.00	111.49	154.15
39	WH	23.97	79.53	165.15
5	CW	6.36	6.79	4.36
7	CW	60.67	0.34	6.55
15	CW	5.60	64.01	15.14
25	CW	4.05	106.97	55.28
30	CW	5.29	29.05	105.51
33	CW	86.99	18.96	99.16
35	CW	61.20	69.29	149.96
40	CW	2.81	76.83	116.20

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